The Role of Serotonin in Pancreatic Acinar Cell Secretion and Regeneration During Pancreatitis

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1. Summary

Pancreatitis is a severe inflammatory disease that causes significant morbidity and mortality. It is currently considered a major risk factor for the development of pancreatic cancer. Acinar cells of the pancreas play an essential role in the initiation of pancreatitis. These cells secrete a variety of digestive enzymes normally in the inactive form (zymogens) to prevent digestion of the pancreas. However, it is now well documented that aberrant secretion and premature activation of zymogens cause cellular damage which triggers the significant inflammatory response characteristic of pancreatitis. Once the disease is initiated, regenerative and repair mechanisms in the pancreas are often unable to restore organ function, leading to organ failure and systemic complications. Causal treatments for pancreatitis are not yet available, thus the current therapeutic interventions aim to relieve pain and to prevent inflammatory relapses. The lack of effective strategies is partially linked to the incomplete understanding of the etiology and pathophysiology of pancreatitis.

In this research project we aimed to investigate the potential signaling pathways underlying both the aberrant enzymatic secretion that initiates the disease, as well as the two main regenerative processes observed in the pancreas, namely progenitor-cell based regeneration and clonal proliferation.

Using a genetically modified mouse model and pharmacological intervention in order to modulate serotonin (5-HT) levels *in vivo*, we demonstrated that the potent bioactive molecule 5-HT regulates zymogen secretion by affecting acinar cell actin-cytoskeleton dynamics. Furthermore, we demonstrated the contribution of 5-HT to the regulation of pro-inflammatory mediators and the subsequent progression of the inflammatory response.

We have also found that 5-HT regulates acinar cell regeneration according to a progenitor-cell based reprogramming but not by clonal proliferation. In particular, we could prove that 5-HT signaling is required for regeneration of acinar cells due to inflammation-mediated cellular injury. In contrast, 5-HT does not contribute to cell proliferation following partial pancreatectomy or mitogenic stimulation.

Significance of the project:

The results of this research show a substantial contribution of 5-HT in the etiopathogenesis of pancreatitis. In particular, we demonstrated that 5-HT promotes pancreatic zymogen secretion, immune cell recruitment and tissue regeneration. Further characterization of 5-HT mediated mechanisms at the molecular level will provide new insights for the management of pancreatitis, possibly limiting pancreatic injury while supporting tissue regeneration.

2. Zusammenfassung

Pankreatitis ist eine schwere entzündliche Krankheit mit hoher Morbidität und Mortalität. Sie wird zurzeit als wichtiger Risikofaktor für die Entstehung eines Pankreaskarzinoms angesehen. Die Azinuszellen des exokrinen Pankreas spielen eine entscheidende Rolle bei der Entstehung von Pankreatitis. Diese Zellen sezernieren eine Vielzahl von Verdauungsenzymen. Um das Pankreas nicht zu verdauen, werden diese Enzyme jedoch normalerweise in einer inaktiven Form (Zymogen) sezerniert. Trotz dieses Schutzmechanismus konnte gezeigt werden, dass abnormale Sekretion und frühzeitige Aktivierung der Zymogene vorkommen kann und Zellschäden verursacht. Die Schäden lösen darauf eine starke entzündliche Reaktion aus, die typisch für eine Pankreatitis ist. Hat die Krankheit einmal begonnen, sind die regenerativen Prozesse und Reparaturmechanismen des Pankreas oft nicht ausreichend, um die Organfunktion wieder vollständig herzustellen. Dies kann zu Organversagen und systemischen Reaktionen führen. Bis heute sind keine ursächlichen Therapieansätze bekannt. Die aktuelle Therapie beschränkt sich auf Schmerzhemmung und der Verhinderung von entzündlichen Rezidiven. Das Fehlen von effektiven therapeutischen Strategien hängt unter anderem damit zusammen, dass die Ätiologie sowie die Pathophysiologie der Pankreatitis noch sehr lückenhaft ist.

In diesem Forschungsprojekt wollten wir die Signalwege untersuchen, welche bei der abnormalen Sekretion und somit bei der Auslösung der Krankheit beteiligt sind. Des Weiteren untersuchten wir die zwei wesentlichen regenerativen Prozesse, namentlich die Progenitorzell-basierte Regeneration und die klonale Proliferation. Durch genetisch modifizierte Mäuse und pharmakologische Interventionen wurden die Konzentrationen von peripherem Serotonin (5-HT) *in vivo* moduliert. So konnte gezeigt werden, dass das potente, bioaktive Molekül 5-HT die Zymogensekretion reguliert, indem es die Dynamik des Aktin-Zytoskeletts beeinflusst. Darüber hinaus wurde der Einfluss von 5-HT zur Regulation der pro-inflammatorischen Mediatoren und der darauffolgenden Entzündungsreaktion untersucht.

Wir haben auch herausgefunden, dass 5-HT die Azinuszell-Regeneration durch eine Reprogrammierung zu Progenitorzellen reguliert. Die Regeneration durch klonale Zellproliferation jedoch, bei der differenzierte Azinuszellen proliferieren, wird nicht durch 5-HT beeinflusst. Insbesondere konnten wir beweisen, dass 5-HT Signale für die Regeneration von Azinuszellen nach entzündlicher Zellschädigung notwendig sind. Im Gegensatz dazu spielt 5-HT keine Rolle bei der Zellproliferation nach partieller Pankreatektomie oder nach mitogener Stimulation.

Signifikanz des Projekts:

Diese Forschungsresultate zeigen, dass 5-HT eine entscheidende Rolle in der Ätiopathogenese der Pankreatitis spielt. Insbesondere haben wir gezeigt, dass 5-HT im Pankreas die Zymogensekretion fördert, Immunzellen rekrutiert und Geweberegeneration begünstigt. Eine weitere Charakterisierung der 5-HT abhängigen Mechanismen auf dem molekularen Niveau werden neue Erkenntnisse für das therapeutische Management der Pankreatitis hervorbringen, welche möglicherweise die Schäden am Pankreas limitieren und gleichzeitig die Geweberegeneration unterstützen.

3. Introduction

Isolated more than 70 years ago, 5-hydroxytryptamine (5-HT), also known by the name of “serotonin”, is a biogenic amine primarily found, in mammals, in the central nervous system (CNS), gastrointestinal tract (GI tract) and blood platelets. Despite its relevance in the CNS, where this neurotransmitter plays a key role in mood, sleep, sex and appetite regulation, 5-HT is produced and released by neurons in a relatively small amount. Over 90% of 5-HT is synthesized, stored and released from a subset of enteroendocrine cells, called enterochromaffin cells (EC), located in the intestinal mucosa. Other modest but nevertheless considerable sources of 5-HT are subtypes of resident granulocytes, called mast cells, pancreatic islets, and enteric neurons of the myenteric plexus, both located in the lamina propria of human and murine bowels [1-3].

3.1 5-HT biosynthesis and metabolism

Biochemically, the synthesis of 5-HT consists in a two-step process. It initiates with the conversion of the essential dietary amino acid L-tryptophan to 5-hydroxytryptophan (5-HTP) by the cytosolic enzyme L-tryptophan hydroxylase (TPH). Subsequently, the enzyme decarboxylase further metabolizes 5-HTP in order to complete the conversion to 5-HT. Although both enzymes are necessary for the synthesis of this bioactive molecule, TPH is considered the rate-limiting enzyme of the biochemical reaction. This enzyme is mostly present in those cells able to produce and store 5-HT. However, many cells contain low levels of TPH, which is relatively unstable. Indeed, minor changes in TPH levels or activity can dramatically alter 5-HT content and serotonergic functions [4, 5].

The first gene coding for the TPH enzyme was found on human chromosome 11 and mouse chromosome 7 [6]. For a long time this gene was thought to be the only TPH gene in the mammalian genome. However, by targeted ablation of this TPH gene (at the present identified as TPH1), the existence of a second TPH isoform, TPH2, was discovered. It is encoded by an additional gene on human chromosome 12 and mouse chromosome 10. The two isoforms share approximatively 71% of similarity, but TPH2 is more selective for tryptophan compared to the isoform 1. Furthermore the two enzymes are expressed in different tissues: TPH1 is mainly present in gut while TPH2 predominates in the brain stem [7, 8]. The existence of these differences ensures a dichotomy of the serotonergic system, which has relevant implications for the regulation of 5-HT production in a spatio-temporal manner.

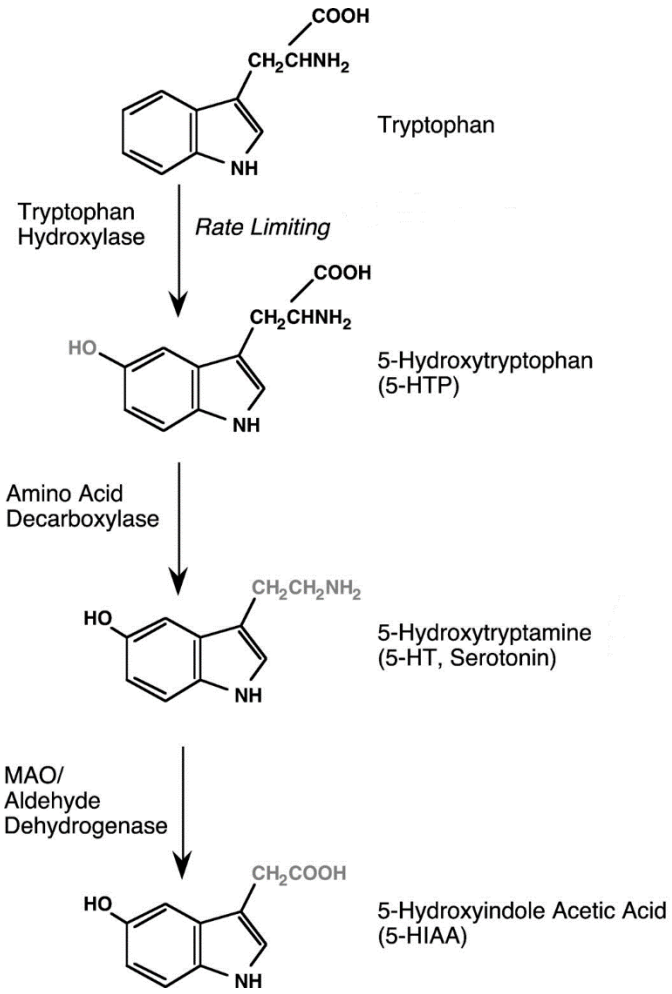

Figure 1. 5-HTsyntesis and metabolism. **Adapted from [9].**

Ninety-nine percent of total body 5-HT is located intracellularly. It is mainly found in presynaptic neurons (CNS) and in platelets (periphery), implying a tight regulation of 5-HT availability. The concentration of 5-HT in tissues depends not only on the rate of synthesis but also on the rate of its metabolism, the latter regulated by the activity of monoamine oxidase (MAO). MAO is a mitochondrial enzyme, ubiquitously expressed, that exists in two major forms, MAO-A and MAO-B. MAO-A metabolizes the biggest amount of 5-HT and it is expressed in most tissues, while MAO-B is found predominantly in blood cells. 5-hydroxyindoleacetic acid (5HIAA) is the metabolite of 5-HT and it is excreted primarily in the urine, within 24 hours in case of exogenous 5-HT administration (Fig.1). Given such a rapid clearance, intracellular storage is the mechanism adopted by the organism to prevent 5-HT from elimination. 5-HT uptake is mediated by the highly selective serotonin transporter (SERT) which is primarily located on presynaptic neurons in the CNS, and on the extracellular membrane of platelets. Once taken up, 5-HT is recycled and stored in small vesicles (reviewed in [10]). The storage in the vesicles is further mediated by vesicular transmembrane monoamine transporters VMAT1 and VMAT2, both expressed in central and peripheral nervous system and in platelet [11] (Fig.2).

Mammals employ 5-HT both as a neurotransmitter in central and peripheral nervous systems, but also as a local hormone in numerous other tissues, including the GI tract, the cardiovascular system and the immune system. The ability of 5-HT to mediate such a plethora of roles implies the necessity of a big variety of receptors. Currently, 18 genes are annotated to encode 14 distinct mammalian 5-HT receptor subtypes.

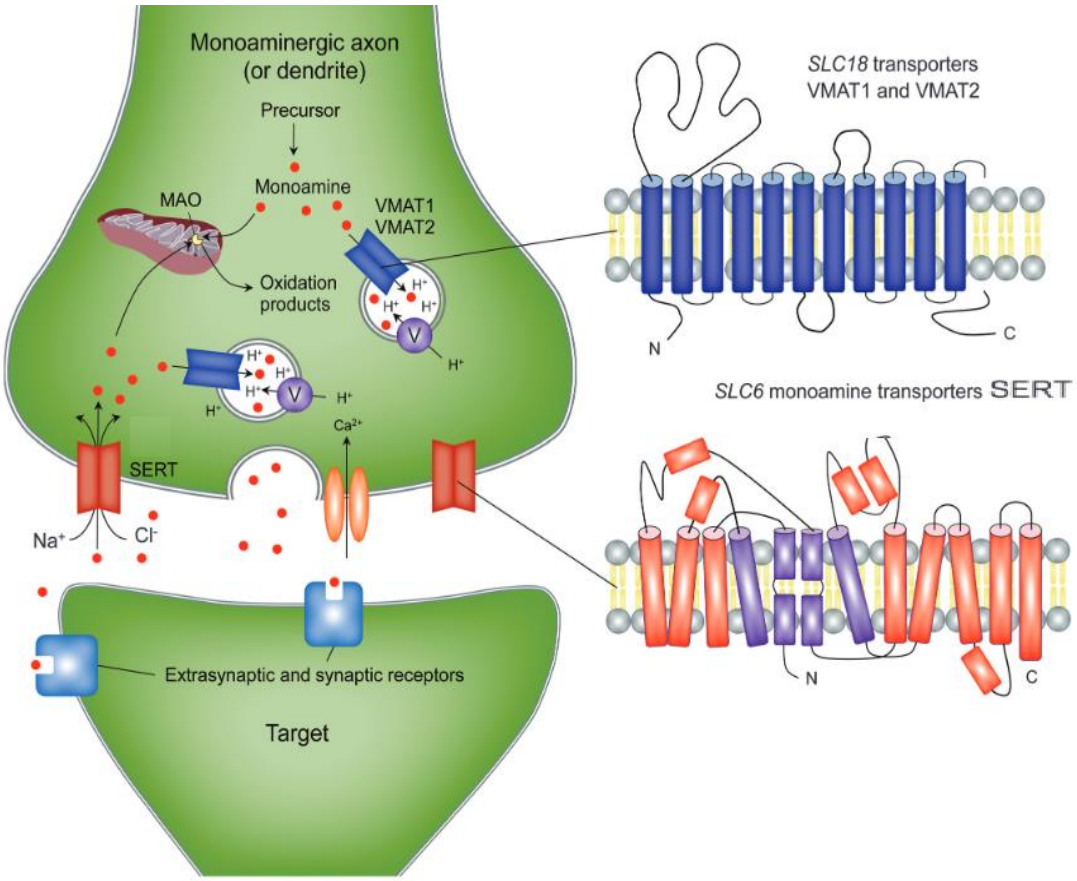

Figure 2. SERT and VMAT-mediated 5-HT uptake in presynaptic terminals. The plasma membrane monoamine transporters and vesicular monoamine transporters (VMATs) are members of the soluble carrier (SLC) superfamily and have a major role in regulation of the homeostasis and effects of 5-HT. SERT is a plasma membrane transporter belonging to the SLC6 family whereas VMATs are members of the SLC18 family. When not sequestered in recycling vesicles, 5-HT is metabolized by the mitochondrial enzyme MAO. **Adopted from [11]**

3.2 5-HT receptors

In the GI tract, 5-HT is released from enterochromaffin cells. It mediates several gastrointestinal functions, such as peristalsis, secretion, vasodilation and perception of pain or nausea. In the brain, the neurotransmitter is produced and released by axon terminals in response to an action potential and then diffuses across the synapse to activate postsynaptic receptors. In both sites, 5-HT signaling is initiated via the activation of a diverse family of receptors. 5-HT receptors are a group of G protein-coupled receptors (GPCRs) and ligand-gated ion channels (LGICs) found ubiquitously in the central and peripheral nervous systems, GI tract, vessels and platelets. They are divided into seven 5-HT receptor families (5-HT_1 to 5-HT_7), most of which have multiple subtypes (e.g. 5-HT_{1A-1F}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B}). The 5-HT receptors are classified according their amino acid homology and their coupling to downstream second messenger pathways. The 5-HT_1 and 5-HT_5 receptor families are generally involved in inhibitory pathways reducing the intracellular levels of cyclic adenosine monophosphate (cAMP) and regulating Ca^{2+} channels. The family 1 is also responsible for the phosphorylation of mitogen-activated protein kinase (MAPK) and initiates growth signaling pathways [12]. On the other hand the families 5-HT_2, 5-HT_4, 5-HT_6, 5-HT_7 activate excitatory pathways increasing intracellular cAMP and PLC activities. Finally, 5-HT_3 mostly regulates negative impulses inducing plasma membrane depolarization and is associated with Na^+/K^+ cation channels. Detailed information regarding 5-HT receptors localization and signaling can be found in the table 1.

Interestingly, structural analyses have recently highlighted the possibility for these receptors to achieve a functional diversity by allelic polymorphisms, splice variants, and the formation of receptor heterodimers [13, 14]. In addition, 5-HT receptors possess sites susceptible to post-translational modification, which can markedly modify their signaling. For instance, prolonged stimulation of 5-HT_{2A} receptors leads to their phosphorylation by G-protein-receptor kinases and/or by protein kinases (PKA and PKC). Phosphorylation of these receptors modifies their cell-surface expression and interaction with G-proteins and subsequent modulation of second messenger levels, which usually leads to blunted signaling [15].

Receptor Subtype	Transduction Mechanism	Localization	Function
1A	↓AC ($G_{i/o}$)	Limbic system (hippocampus, lateral septum, cortical areas), mesencephalic raphe nuclei	Hyperpolarization, modulation of neurotransmitter release, anxiolysis, hypothermia, hyperphagia
1B	↓AC ($G_{i/o}$)	Basal ganglia, striatum, amygdala, trigeminal ganglion, vascular smooth muscle	Autoreceptor, locomotion, hypophagia, hypothermia, modulation of neurotransmitter release, vasoconstriction
1D	↓AC ($G_{i/o}$)	Basal ganglia, hippocampus, cortex, spinal cord, vascular smooth muscle	Autoreceptor, modulation of neurotransmitter release
1E	↓AC ($G_{i/o}$)	Cortex, caudate putamen, claustrum, hippocampus, amygdala	Unknown
1F	↓AC ($G_{i/o}$)	Hippocampus, cortex, dorsal raphe nucleus, uterus	Speculative role in visual and cognitive function
2A	↑ PLC	Forebrain, caudate nucleus, nucleus accumbens, hippocampus, olfactory tubercle, vascular smooth muscle, blood platelets	Neuronal depolarization, head twitch, hyperthermia, modulation of neurotransmitter release smooth muscle contraction, platelet activation
2B	↑ PLC	Brain, stomach fundus (rat), gut, heart, kidney, lung	Contraction of the stomach fundus, anxiety
2C	↑ PLC	Choroid plexus, cortex, limbic system, basal ganglia	Hypolocomotion, hypophagia, penile erection, hyperthermia, anxiety, ↓ noradrenalin and dopamine release
3	Ion channel (Na^+, K^+, Ca^{2+})	Dorsal vagal complex, hippocampus, amygdala, caudate, cerebral cortex, heart, intestines	Anxiety, cognition, pain , reward/withdrawal, vomiting reflex, vasodilation, intestinal tone and secretion
4	↑ AC (G_s)	Cerebral cortex, limbic areas, hippocampus, colliculus, intestines	Learning and memory, visual perception, anxiety, motor coordination, arousal, smooth muscle relaxation, modulation of neurotransmitter release
5A	↓ AC ($G_{i/o}$)	Amygdala, hippocampus, caudate nucleus, cerebellum, hypothalamus, thalamus, substantia nigra, spinal cord	Modulation of exploratory behavior and locomotion
6	↑ AC (G_s)	Striatum, olfactory tubercles, nucleus accumbens, hippocampus, stomach, adrenal glands	Memory and learning, modulation of neurotransmitter release
7	↑ AC (G_s)	Thalamus, hypothalamus, hippocampus, cerebral cortex, amygdala, GI and vascular smooth muscle, heart	Circadian rhythms, smooth muscle relaxation, nociception, hypotension, modulation of REM sleep, learning and memory, LH release

Table 1: Adapted from Tocris web site

3.3 5-HT transporter

5-HT bioavailability and functions are tightly regulated by a combination of reuptake mechanisms, feedback loops, and metabolizing enzymes [16]. The maintenance of a robust 5-HT signaling largely depends on the precise control of extracellular 5-HT levels. The 5-HT uptake transporter SERT is considered the primary molecule responsible for inactivating 5-HT signaling in the CNS and GI tract.

SERT belongs to the neurotransmitter/sodium symporter (NSS) family, according to a Na^+/Cl^--dependent mechanism and it mediates the uptake of 5-HT across the membrane of both neuronal and non-neuronal cells. SERT has been found in two different isoforms which differ not only in length but also in distribution. The peripheral SERT isoform is more abundant and shorter compared to the one found in CNS. Its transcription initiates downstream from the starting site and a tissue specific regulatory sequence, located within intron 1, determines its expression and abundance in the periphery [17]. Generally, SERT function is rapidly inhibited in response to acute depletion of intracellular Ca^{2+}, inhibition of calmodulin, Src-kinase, p38 MAPK and activation of PKC. Otherwise, increased intracellular Ca^{2+}, activation of NOS/cGMP and MAPK pathways stimulate SERT activity. Importantly, SERT gene expression, activity and clearance are directly or indirectly regulated both by 5-HT receptor signaling and 5-HT extracellular availability. In synaptosomes, for instance, it was demonstrated that ablation, either genetic or pharmacologic, of the 5-HT_{1B} receptor leads to a reduction of SERT gene expression. On the other hand, SERT activity depends on 5-HT_{2B} receptor signaling. In the absence of external 5-HT, 5-HT_{2B} receptor ensures SERT phosphorylation to basal level and maximal 5-HT uptake. In the presence of 5-HT, the 5-HT_{2B} receptor promotes hyper phosphorylation of SERT, impairing the electrochemical gradient necessary for the uptake of 5-HT.

Uptake of 5-HT was considered for long time a scavenger system adopted by the cells either to regulate 5-HT signaling, but also to store or metabolize excessive levels of extracellular 5-HT. However, in 2003 a new biological function was attributed to 5-HT taken up by SERT. When internalized, 5-HT is covalently bound to target proteins and thus modifies their activity. Accordingly, this post-translational modification was named *serotonylation*. Multiple physiological roles have been identified since its description and deregulated serotonylation was shown in the etiology of bleeding disorders, primary pulmonary hypertension and diabetes (reviewed in [18]).

5-HT uptake mechanism is highly conserved among the species and it takes places in several cell types regardless their ability to synthetize 5-HT. During mammals development, for example, 5-HT storage is firstly observed in non-neuronal sites such as heart, cranial mesenchyme and notochord, and curiously, it takes place in the CNS only during post-natal development [19].

Regarding the CNS, 5-HT uptake is observed not only in those neurons capable of 5-HT synthesis, but also in the non-monoaminergic one. This peculiarity has risen up several questions regarding the functional role of 5-HT internalization during CNS development, and an exhaustive explanation is still needed. One fascinating hypothesis suggests that during cranial development, non-monoaminergic neurons transiently express SERT in order to internalize 5-HT which is afterward utilized as a "borrowed neurotransmitter" to modulate extra-neuronal signaling. Alternatively, the uptake serves to control the extracellular level of 5-HT avoiding excessive receptor stimulation and guaranteeing the correct 5-HT gradient during brain development [20].

The example of 5-HT trafficking in the periphery highly supports each of the mentioned hypothesis. Platelets, for instance, capture 5-HT from the bloodstream and store it in dense-core vesicles. Also in this cell type, 5-HT is not produced by platelets but it is rather used as a borrowed transmitter released in case of tissue damage or allergic reactions which imply platelet activation. In other cases, such as intestinal crypt cells or endothelial cells, the expression of SERT and the function of heterologous 5-HT uptake serve as a clearance pathway aiming to inactivate the 5-HT produced and released by neighboring cells [21, 22] (Fig.3). A better understanding of the signaling pathways influencing SERT expression and functions will shed light on the myriad of pathways through which this neurotransmitter regulates central and peripheral activities.

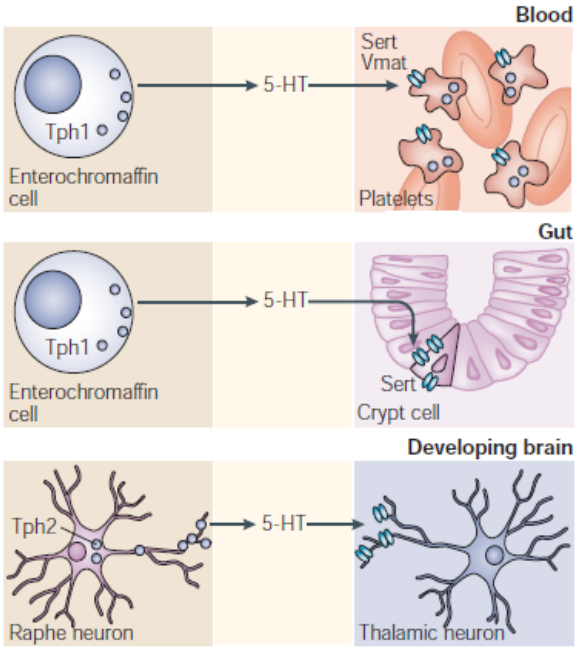

Figure 3. Circulation of 5-HT between cells that produce 5-HT and those that capture it: in the blood, circulating 5-HT derives essentially from the enterochromaffin cells. Plasma 5-HT is captured and concentrated by platelets, which release it on stimulation. In the gut, enterochromaffin cells release 5-HT that is captured by the crypt cells, which metabolize the amine. In the developing brain, 5-HT is produced by raphe neurons and is captured by thalamic axons, which store it in synaptic vesicles. **Adapted from [23].**

3.4 5-HT signaling in the brain

In the adult CNS, 5-HT is produced by a moderate-size cluster of neurons originating in the raphe nuclei located in the midline of the brainstem. Raphe nuclei consist in a heterogeneous population of neurons with distinct morphologies, projections and neurochemical characteristics, among which the serotonergic neurons are the most representative. These neurons are functionally diverse according to their localization: descending medullary raphe projections influence spinal and brainstem mechanisms and are mostly involved in the central modulation of pathologic pain syndromes occurring in the bowel [24]. By contrast, the ascending raphe projections are implicated in the regulation of mood, sleep, sex, appetite, or most generally, all human behavior [25]. Human behaviors result, indeed, by the combined signaling of several 5-HT receptors, expressed in multiple brain regions, which simultaneously modulate several processes. For example, anxiety-like behaviors are regulated primarily by 5-HT_{1A} and 5-HT_{2C} receptors. However, the 5-HT_{2C} receptor, beside anxiety, regulates also reward, appetite, locomotion, and energy balance. For this reason, pharmacological treatment targeting 5-HT_{2C} receptor may affect multiple and apparently unrelated behavioral processes (reviewed in [26]).

An intriguing and at the same time puzzling issue regards the role of 5-HT in brain development. Studies on murine models reported that during this stage, receptors, transporter and degrading enzymes of 5-HT appear much earlier than the serotonergic innervation [27]. A new rising hypothesis postulates that during early embryogenesis, a tryptophan metabolic pathway in the maternal placenta constitutes the extra-embryonic source of 5-HT, which influences not only the fetal brain development (via specific receptor signaling) but also its long-term functions [28]. A typical example is the 5-HT_{1A} receptor which is expressed in the raphe nuclei and in the hippocampus during early developmental stage and it is stimulated by maternal 5-HT. After the gestation, this receptor is transiently expressed in hippocampal dendritic spines and is responsible for their post-natal elongation [29]. Finally, in adulthood, its signaling is associated with anxiety-like behavior [26, 30].

Differently, the 5-HT_{2A} receptor does not actively take part to embryonic brain development since its early ablation is not associated to any developmental CNS abnormalities. Its activation is rather crucial during the post-natal phase for the maturation of phrenic motor neurons, which are indispensable for breathing and control the functions of the diaphragm. Nevertheless, the 5-HT_{2A} receptor is the most expressed subtype in nearly all adult brain regions and its over-expression on post-synaptic neurons is associated to depressive behavior [31].The examples reported above emphasize the concept that 5-HT receptor expression and activities cannot be limited to a specific brain region or developmental stage but rather they have to be considered highly dynamic and represent a particularly complex signaling system.

3.5 5-HT signaling in the gastrointestinal tract

Although the enteric nervous system (ENS) has been defined the “second brain” due to its functional autonomy, in terms of anatomy it is intrinsically connected to the CNS via motor and sensory fibers (*vagus nerves*) of the parasympathetic nervous systems.

Since its discovery, 5-HT was proposed to be one of the key neurotransmitter bridging the two systems during gut activity. The exact mechanisms through which 5-HT connects these two compartments have yet to be definitely established but scientists tend to favor a neuro-endocrine cross-talk in which enterochromaffin cells act as sensory transducers which respond to increased intraluminal pressure (due to food intake) by secreting 5-HT that locally activates serotonergic sub-mucosal neurons. This hypothesis, formulated already in the early 1980 when 5-HT was found to induce a propulsive motor function in mammalian intestine [32], was later on corroborated by several analyses showing that ascending contractile and descending relaxant limbs of peristaltic reflex were depending respectively on 5-HT_3 and 5-HT_4 receptors located on enteric afferent neurons [16, 33-36]. In the last decades, however, follow up studies have clarified that gut-derived 5-HT is not necessary to initiate this reflex. In fact, general Tph1 knock out mice, although deficient in gut-derived 5-HT, do not show any dramatic alteration in intestinal motility suggesting that enterochromaffin-derived 5-HT does not evoke peristaltic reflexes, but only modulate them [36, 37]. On the other hand, lack of TPH2, whose expression in gut is minimal and occurs exclusively in enteric neurons, results in slower gastrointestinal transit time, intestinal propulsion and colonic motility and faster gastric emptying, suggesting that serotonergic neurons are more critical than enterochromaffin cells in regulating gut motility. Of note, lack of TPH2 in gut is primarily associated to a disrupted myenteric plexus formation, which normally takes place during embryogenesis. For this reason dysmotility found in TPH2 knockout mouse may reflect either an alteration in serotonergic neurotransmission and/or changes in the 5-HT “microsystem” (release/uptake, receptor signaling) due to loss of neurons [37].

Not only mechanical stimuli, such as augmented luminal pressure, but also chemical stimuli were found to specifically modulate 5-HT release and signaling in the ENS. In presence of glucose, enterochromaffin cells release 5-HT that excites the receptors located on afferent nerve terminals to evoke gastric motility and delay gastric empting [38]. In case of lipid intake, 5-HT receptors and cholecystokinin (CCK) receptors are activated in order to signal back the sense of satiety [39].

5-HT has also the ability to coordinate, via neuronal and not neuronal pathways, secretory processes that culminate into the release of electrolytes, mucus, fluids and enzymes by intestinal epithelial cells [40] and pancreatic cells [41, 42].

3.6 5-HT-mediated pancreatic secretion

Situated in the retroperitoneal cavity, the pancreas is an organ which acts both as an endocrine gland secreting hormones, and as an exocrine gland secreting digestive enzymes. The endocrine part, which represents approximatively ~5% of the organ, is organized in functional units called islets of Langerhans where five hormone-secreting cell types, called β, α, δ, ε and PP- cells, are clustered. A dense network of capillaries crisscrosses the islets of Langerhans, and constitutes a physical connection between the endocrine cells and the surrounding blood vessels. This proximity in terms of space ensures a prompt release of hormones, such as insulin, glucagon, and somatostatin, in response to blood glucose level variations. Insulin secretion, in particular, was recently found to be regulated by a non-neuronal 5-HT signaling. 5-HT is synthesized within β-cells by TPH1 enzyme [3], is stored together with insulin in β-granules and it actively takes part to insulin secretion via autocrine/paracrine pathways. Paulmann and colleagues have demonstrated that lack of peripheral 5-HT in Tph1$^{-/-}$ mice leads to a significant reduction of β-cells insulin secretion, increased insulin resistance and appearance of a diabetic type II phenotype. Pharmacological administration of 5-HT in these mice rescued insulin secretion and reverted the resistance. In his study, Paulmann elegantly demonstrated that, under physiological conditions, intracellular rather than extracellular 5-HT is crucial for hormonal secretion. After being taken up by SERT, localized on β-cells membranes, 5-HT binds and thus activates small GTPases, namely Rab3a and Rab27a, which actively promotes the β-granules secretory process [43].

Additional investigations performed on pregnant animal models displaying temporary gestational diabetes, further emphasized the fundamental role of 5-HT in β-cells secretion. Upon lactogenic signaling, Tph1 expression and activity increase dramatically, islets increase their cell mass and a temporary insulin resistance arises [44-46]. Different 5-HT receptor subtypes were found to directly regulate β-cell proliferation and glucose-induced insulin secretion during the gestational time [44, 47]. It is worth to mention that in this particular condition, granule exocytosis resulted from an extracellular 5-HT signaling via cell-surface receptor and no involvement of intracellular 5-HT was detected [47]. Collectively, the above mentioned studies on β-cell secretion highlight that the 5-HT microsystem is so complex that one cell function, such as granule exocytosis, depends on multiple pathways.

The exocrine or “digestive” part of the organ is composed of ductal and acinar cells (Fig.4). In the adult pancreas, ductal cells represent the minor cell type and, besides forming a network that delivers enzymes from acini into the digestive tract, they produce bicarbonate that neutralizes stomach acidity and balance the pH of pancreatic juice [48]. Notably, a non-neuronal 5-HT receptor-mediated signaling was proposed to negatively control the

bicarbonate secretion, data recently supported by the evidence that lack of peripheral 5-HT results in increased ductal bicarbonate secretion [49].

Acinar cells are highly polarized units that produce, store and release digestive enzymes necessary for proper food digestion and absorption. These enzymes, namely amylase, lipase and proteases, are the major constituent of the so called pancreatic juice. These enzymes are secreted through the apical membrane of acinar cells into small branched intercalated ducts that drain this fluid through a dense network of tubular structures eventually joining the main pancreatic duct (Fig.4). Pancreatic digestive enzymes are produced in the endoplasmic reticulum as precursor molecules (zymogens) and stored into membrane-enclosed compartments called zymogen granules. Specifically, most zymogens contain an inhibitory domain that keeps them inactive until a cascade of enzymatic cleavage steps removes them. Trypsinogen, the precursor form of trypsin, is committed to initiate the proteolytic cascade and thus converts the zymogens not yet activated. Physiologically, the activation of trypsinogen into its active form is carried out by the brush-border enzyme enterokinase (belonging to enteropeptidase family) in the small intestine.

As discussed above, most of the pancreatic functions are controlled both by neuronal and non-neuronal pathways. In line with this concept, secretion of digestive enzymes is the result of the stimulation of both pathways. Hormones such as secretin, CCK, gastrin releasing peptide (GRP), substance P, vasoactive intestinal peptide (VIP) and acetylcholine bind their corresponding acinar receptors and, through interaction with G-proteins, trigger the process of receptor-mediated secretion. At the same time, enzymatic secretion is also induced by the synergistic stimulation of CCK and 5-HT receptors localized on vagal afferent fibers [41, 50].

More than 30 years ago, it was proposed that pancreatic rat acinar cells could produce and release a little amount of 5-HT and, similarly to what happens in β-cells, 5-HT was co-localized in mature amylase- containing zymogen granules [51]. However, since this preliminary observation, the exact role of 5-HT in acinar cells zymogen granules has not been clarified.

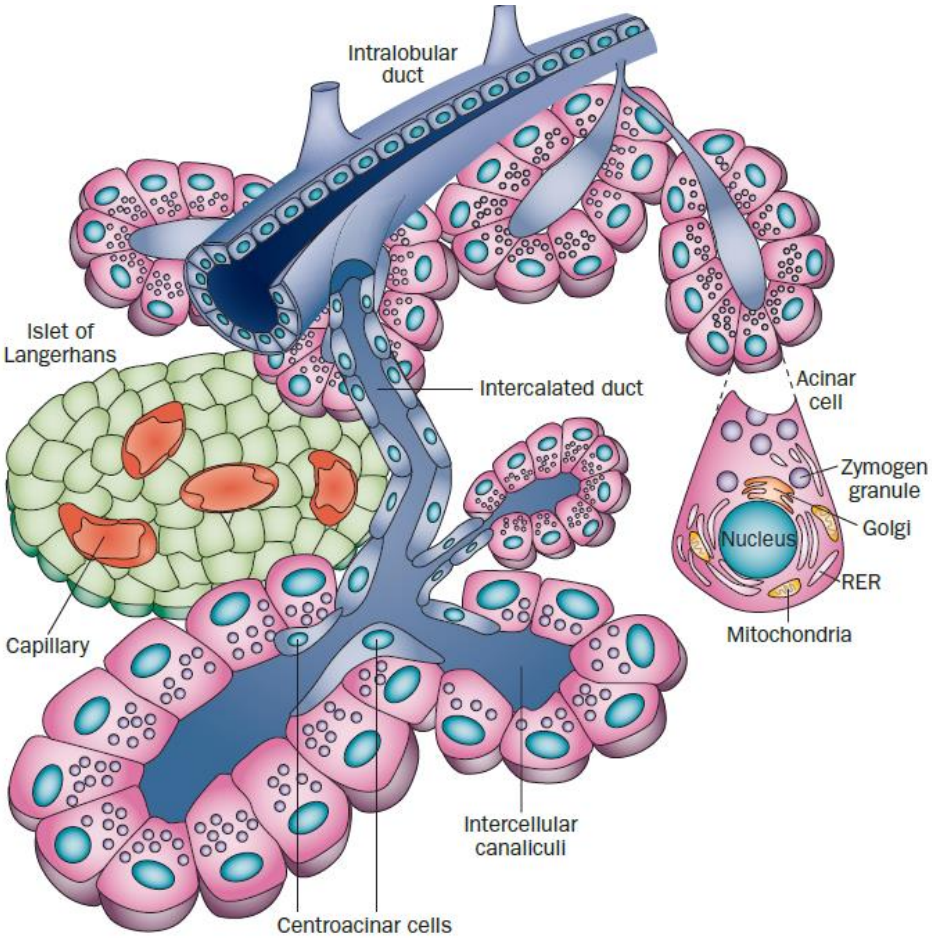

Figure 4. The cellular components of the pancreas. The pancreas consists of endocrine cells organized in clusters called Islets of Langerhans, which contain multiple endocrine cell types including the β cells that secrete insulin. The exocrine pancreas is composed of acinar and ductal cells. Pancreatic acinar cells are organized in small structures called acini that surround central lumen open to the duct system. Pancreatic acinar cells produce, store and secrete enzymes necessary for food digestion and absorption. Enzyme secretion occurs through the apical membrane of the acinar cell into small intercalated ducts that are directly connected to increasingly larger intralobular ducts that join the main pancreatic duct. **Adopted from [52]**

3.7 Aberrant pancreatic secretion and pancreatitis

Under physiological conditions, pancreatic acinar cells are constantly subjected to high levels of stress due to their elevated protein synthesis, packaging, secretion and also because unexpected premature activation of newly synthesized zymogens may damage intracellular components requiring a prompt recovery. In this case, repairing systems, such as the presence of intracellular trypsin inhibitors or proteases degrading the activated zymogens, normally prevent cellular damage. Whenever the mentioned protective mechanisms are disrupted, zymogens are activated intracellularly and acinar cell auto-digestion initiates. This type of injury evokes an inflammatory response.

Clinically defined as “pancreatitis”, pancreatic inflammation is classified either as acute or chronic according to the evoked inflammatory response. Acute pancreatitis is associated either to a mild and localized inflammation, which generally resolves in a relatively short time, or to a more severe and systemic one, which leads to multiple organ dysfunction and death. On the other hand, chronic damage is mostly characterized by recurrent phases of acinar cell destruction and replacement, either with fibrotic or fat tissue, which culminates in exocrine insufficiency.

A number of risk factors are associated with the onset of pancreatitis, namely bile duct obstruction by gallstones, alcohol abuse, smoke, high fat diet, infection or trauma; however a clear pathogenic mechanism is not yet established. High levels of intracellular activated trypsin are known to exacerbate acute pancreatitis causing acinar cell apoptosis [53] or autophagy [54]. However, more recent and controversial evidence suggest that the intracellular trypsin activation is sufficient to initiate pancreatitis but it does not determine the severity of the local inflammation [55, 56]. In fact, recent studies report, that extracellular but not intracellular activated trypsin, is able to stimulate the pro-inflammatory gradient [55, 57, 58], suggesting that trypsin not only has to be aberrantly activated but it must be secreted ectopically in the interstitial space in order to dramatically contribute to tissue injury.

The precise molecular mechanisms regulating the ectopic interstitial secretion during pancreatitis remain largely unclear. It was proposed to be largely dependent on cytoskeletal remodeling in acinar cells. Pancreatic acinar cells, like other secretory cells, have a dense web of actin filaments immediately beneath their plasma membrane, better known as terminal web. Under resting conditions, this structural component prevents secretory granules from reaching their exocytic destination acting as a physical barrier. During granule exocytosis, this web is temporarily perturbed allowing granules containing enzymes to dock and fuse with the apical plasma membrane and finally release their content into ductal lumens. Dysregulation of this mechanism is currently believed to be a contributing cause of clinical acute pancreatitis. Experiments performed on isolated rodent acinar cells revealed that stimulation of enzymatic secretion with supramaximal doses of CCK, or its synthetic

analogue cerulein, induces a massive disruption of terminal actin web with the consequent block of apical secretion and a redirection of exocytosis toward the baso-lateral cell membrane [59]. It was also clarified that upon CCK stimulation both actin remodeling and granule exocytosis were mediated by small GTPases such as Rho, Rac and Rab [60, 61].

Although ectopic secretion is certainly crucial for initiation of pancreatitis, it is worth to mention that several other impaired cellular processes may independently initiate tissue damage. Endoplasmic reticulum stress due to excessive protein synthesis, ROS production or impaired protein folding/packaging, stimulates the secretion of pro-inflammatory cytokines, which locally recruit and activate inflammatory cells. Shortly after the initial insult, acinar cell secrete pro-inflammatory factors like TNF-α, IL-6, IL-1β, and MCP-1, recruiting firstly neutrophils and then macrophages which exacerbate the tissue injury. In concomitance with this inflammatory cell influx, acinar cells initiate a proliferative process. Surrounded by inflammatory cells, and possibly influenced by their secretome, a portion of acinar cells undergo a trans-differentiation program. At first, a general loss of enzymatic content is observed, accompanied by a robust down regulation of genes responsible for zymogen production. Subsequently, a portion of acinar cells acquire a new identity losing their polarity in favor of a more cuboid shape, shrinking and remodeling their cytoskeletal architecture and becoming highly proliferative cells organized into duct-like structures. Furthermore, these structures better identified as acinar-to-ductal metaplasia (ADM), transiently express not only genes typical of pancreatic embryogenesis, namely Hnf6, Pdx1, Notch1 and Hes1, but also those normally restricted to adult duct cells, such as cytokeratin 19 (CK19) and Sox9 [62-64]. In the absence of further inflammatory relapses, re-differentiation into functional acinar cells occurs rapidly within one week after cessation of the insult.

Histological analysis on human specimens strongly suggests that the above mentioned regenerative process is likely to happen also during human pancreatitis. Damaged human pancreata, surgically resected upon pancreatitis, display increased proliferation of intact acini and histological changes consistent with ongoing regeneration, including ADM formation.

Of note, concomitant acinar trans-differentiation and proliferation are easily reproducible in rodents using the model of cerulein-induced pancreatitis and their extent can be prolonged or intensified simply by modulating the drug treatment [65]. Taken together, these observations suggest that the process of trans-differentiation represents a protective mechanism adopted by acinar cells in response to aberrantly secreted enzymes.

The entire proposed mechanism is summarized in the Fig.5 reported below.

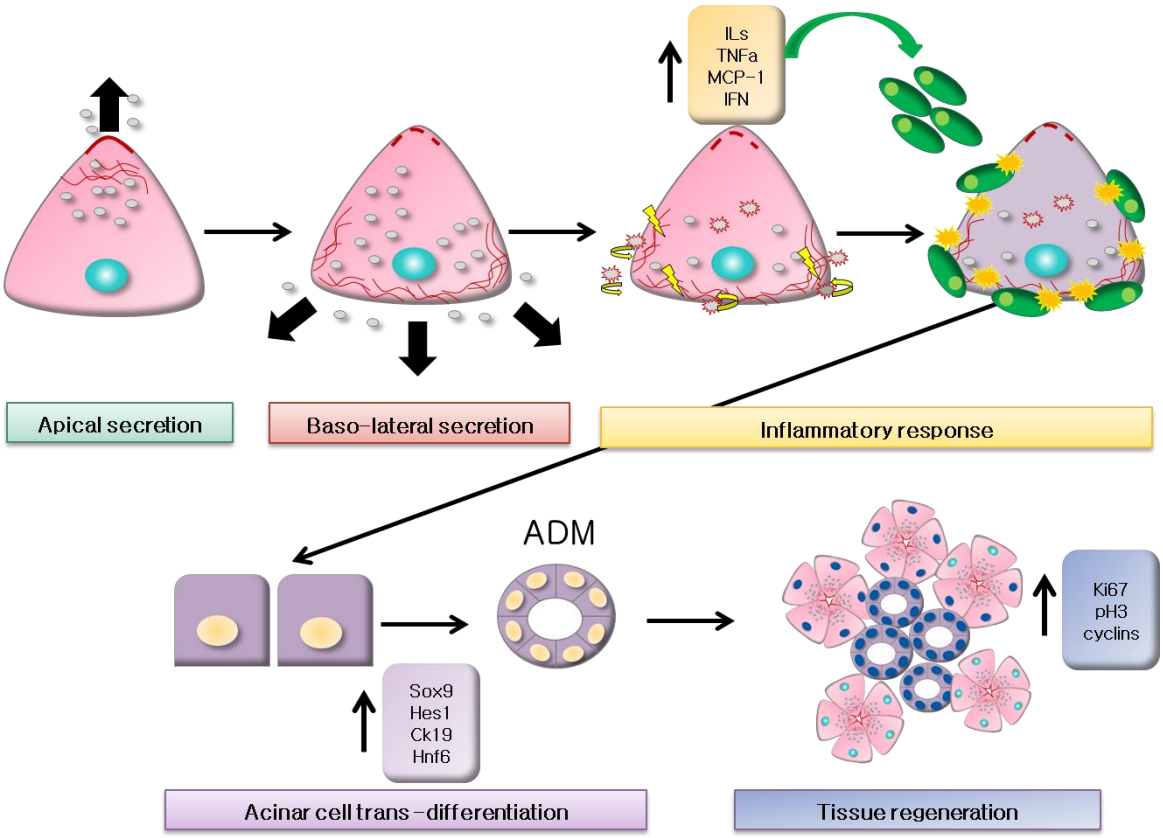

Figure 5. Schematic representation of pancreatitis progression. Please note as during acinar cell trans-differentiation phase, nuclei express markers of pancreatic embryogenesis and markers restricted to adult ductal cells. During the regenerative phase, both intact acini and trans-differentiating foci become highly proliferative.

4. Aim of the project

Early in the course of pancreatitis, premature activation and ectopic interstitial secretion of pancreatic digestive enzymes lead to acinar cell injury and necrosis characteristic of this disease. This early event culminates first into a severe inflammation and then, in the absence of additional insults, into tissue recovery.

Although several information were gained in the last decades regarding the subsequent phases of pancreatitis, the molecular mechanisms regulating aberrant acinar cell secretion and tissue recovery following tissue damage remain largely unclear.

In the search of the molecular mechanisms regulating both pancreatic acinar cell ectopic secretion and regeneration, we focused our attention on 5-HT, a potent bioactive molecule recently found to modulate cell secretion but also well known for its mitogenic properties.

Hence, we hypothesize that 5-HT may play a decisive role in two fundamental phases of pancreatitis, namely acinar cell secretion and acinar cell regeneration.

In particularly we aim to:

1) Investigate whether 5-HT regulates zymogen secretion in pathological conditions. To address this question we analyzed acinar cell secretion in a genetically modified mouse model lacking tryptophan hydroxylase 1 ($Tph1^{-/-}$), the rate limiting enzyme for the synthesis of peripheral 5-HT. Pathological consequences were evaluated in adult animals (8-10 weeks of age) during the course of pancreatitis caused by multiple intraperitoneal administration of cerulein. In addition, AR42J cell, a rat acinar cell line with remarkable secretory characteristics, and freshly isolated acinar cells were utilized to investigate the role of intracellular 5-HT on enzymatic dynamic.

2) Analyze whether 5-HT acts as a mitogenic factor for pancreatic acinar cells, thereby promoting pancreatic tissue regeneration. To answer this question, we compared three types of regenerative stimuli, namely cerulein-induced pancreatitis, partial pancreatectomy and thyroid hormone supplementation, in$Tph1^{-/-}$ mice and in wild-type mice supplemented with 5-HT precursor. In particular, we evaluated whether circulating 5-HT was necessary for acinar cell proliferation in a context of inflammation-mediated damage and tissue loss following surgery. Furthermore, we also evaluated whether 5-HT availability could influence the proliferation of healthy and already proliferating acinar cell independently stimulated with a second mitogenic factor.

5. Manuscript A

Serotonin regulates amylase secretion and acinar cell damage during murine pancreatitis

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Contribution: This study represents a part of my work completed during the first and second years of the PhD. I mostly contributed to this paper characterizing actin and Rac1 localization, and performing qPCR analysis on immunity markers. I also contributed to the manuscript revision.

Serotonin regulates amylase secretion and acinar cell damage during murine pancreatitis

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Short Title: 5-HT in Pancreatitis

Abbreviations: AP, acute pancreatitis; CP, chronic pancreatitis; 5-HT, 5-hydroxytryptamine; 5-HTP, 5-hydroxytryptophan; TPH, tryptophan hydroxylase; SERT, serotonin reuptake transporter; MCP, monocyte chemoattractant protein; IL, interleukin; TNF, tumor necrosis factor; HMGB, high mobility group box.

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Keywords: 5-HT; MCP-1; zymogen secretion; pancreatitis

Word count: 3926

Abstract

Objective: Serotonin (5-hydroxytryptamine, 5-HT) is a potent bioactive molecule involved in a variety of physiological processes. In this study, we analyzed whether 5-HT regulates zymogen secretion in pancreatic acinar cells and the development of pancreatic inflammation, a potentially lethal disease whose pathophysiology is not completely understood.

Methods: 5-HT regulation of zymogen secretion was analyzed in pancreatic acini isolated from wild-type or tryptophan hydoxylase-1 knock-out ($TPH1^{-/-}$) mice, which lack peripheral 5-HT, and in amylase-secreting pancreatic cell lines. Pancreatitis was induced by cerulein stimulation and biochemical and immunohistochemical methods were used to evaluate disease progression over two weeks.

Results: Absence and reduced intracellular levels of 5-HT inhibited the secretion of zymogen granules both *ex vivo* and *in vitro* and altered cytoskeleton dynamics. In addition, absence of 5-HT resulted in attenuated pro-inflammatory response after induction of pancreatitis. $TPH1^{-/-}$ mice showed limited zymogen release, reduced expression of the pro-inflammatory chemokine MCP-1 and minimal leukocyte infiltration compared with wild-type animals. Restoration of 5-HT levels in $TPH1^{-/-}$ mice recovered the blunted inflammatory processes observed during acute pancreatitis. However, cellular damage, inflammatory and fibrotic processes accelerated in $TPH1^{-/-}$ mice during disease progression.

Conclusions: Our results identify a 5-HT-mediated regulation of zymogen secretion in pancreatic acinar cells. In addition, they demonstrate that 5-HT is required for the onset but not for the progression of pancreatic inflammation. These findings provide novel insights not only into the normal physiology of pancreatic acinar cells, but also into the pathophysiology of pancreatitis, with potential therapeutic implications.

What is already known about this subject?

- Impaired zymogen secretion is a central event in the pathophysiology of pancreatitis.
- Alterations in actin cytoskeleton are involved in the secretory defect observed in acinar cells.
- Serotonin modulation of zymogen secretion has not yet been proposed.

What are the new findings?

- We provide evidence that serotonin regulates zymogen secretion and actin dynamics in pancreatic acinar cells.
- $TPH1^{-/-}$ mice defective in peripheral serotonin showed reduced zymogen secretion accompanied by limited inflammatory response following induction of acute pancreatitis.
- Lack of serotonin increased pancreatic cellular damage, inflammation and fibrosis during pancreatitis progression toward a chronic stage.

How it might impact on clinical practice in the foreseeable future?

- Comprehensive characterization of the early events during development of pancreatitis is critical to understand the pathophysiology of the diseases and to supply a strong foundation for the discovery of new treatments.
- Our findings reveal the pivotal role 5-HT plays in the secretory processes in pancreatic acinar cells. Absence of this molecule influences both the onset and the progression of cerulein-induced pancreatitis.
- Collectively, our findings provide new insights into pancreatic pathophysiology and highlight the potential and limitations of therapeutic strategies based on 5-HT-mediated pathways during pathological inflammatory events.

Introduction

Acute pancreatitis (AP) is an inflammatory disorder of the pancreas, which in its severe form is associated with a mortality rate of 15-25%. [1] The etiologic factors involved in the initiation and aggravation of AP are still poorly understood, and the treatment of the disease is limited to supportive therapy.[2] Thus, efforts to understand the pathophysiology of this disorder and to develop clinical strategies to attenuate the disease progression are imperative.

Results from human studies and murine experimental models indicate that activation of zymogen proteases inside acinar cells, a central and early event in pancreatitis, is not sufficient to induce acinar cell injury but must be coupled with a reduction and redirection of zymogen secretion from the apical to the basolateral membrane of acinar cells, thus highlighting the importance of secretion in disease initiation (reviewed in [3, 4]). While the precise molecular mechanisms regulating this aberrant secretion remain largely unclear, ex vivo studies on isolated pancreatic acini indicated that disturbance of apical actin cytoskeleton dynamics mediated by cellular small GTPases are implicated in the process.[5, 6] To further investigate the mechanisms regulating zymogen secretion both in physiological and pathological situations, we analyzed whether serotonin (5-hydroxytryptamine, 5-HT) regulates the secretory processes in pancreatic acinar cells. The generation of tryptophan hydoxylase-1 knock-out ($TPH1^{-/-}$) mice in which 5-HT is absent in the peripheral circulation, but remains unaffected in the central nervous system, [7] has been a pivotal instrument to reveal the numerous roles of 5-HT outside the nervous system. These extend from modulation of organ regeneration, as recently demonstrated by our group in liver following hepatectomy,[8] to regulation of inflammatory cell activity.[9, 10, 11, 12, 13] Importantly, 5-HT has also been shown to regulate secretion in two cell types, namely platelets,[14] and pancreatic β cells.[15] In both cell systems, 5-HT exerted its regulatory action by modifying and thus activating intracellular proteins, including small GTPases, via a transglutaminase-mediated process known as serotonylation. In addition, recent evidence indicates that serotonylation is found on cytoskeletal proteins including actin and regulates contractility in vascular smooth muscle,[16, 17] and membrane architecture in intestinal epithelial cells.[18] This suggests that 5-HT is also likely to regulate structural processes.

The observation that 5-HT modulates the activity of small GTPases and actin cytoskeleton, and that both these elements are involved in the secretory process of pancreatic acinar cells, lead us to hypothesize that 5-HT may be a key factor in regulating zymogen secretion both in physiological and pathological situations. To address this question we analyzed acinar cell secretion and pathophysiological consequences in the pancreas using 5-HT deficient $TPH1^{-/-}$mice.

Materials and methods

Animal experiments

All animal experiments were in accordance with swiss federal animal regulations and approved by the cantonal veterinary office of Zurich. Pancreatitis was induced in adult (8-10 weeks of age) wild-type (WT) C57BL/6 (Harlan Laboratories, Horst, The Nederlands) and $TPH1^{-/-}$ mice,[7] on a C57BL/6 background (own breeding) via intra peritoneal (i.p.) administration of six hourly injections of 50 µg/kg cerulein over a two week period, as described.[19] Reloading with the serotonin precursor 5-HTP was performed with 20 mg/kg 5-HTP by two sub cutaneous (s.c.) injections per day, 12 hours apart. The treatment was performed on alternate days, commencing one day before the cerulein treatment; control animals received 0.9% NaCl injections.

Isolation of pancreatic acini

Pancreatic acini from WT and $TPH1^{-/-}$ mice were prepared by enzymatic digestion with collagenase followed by mechanical shearing, as described.[20] Acini were pacified in Kaighn's modified Ham's F-12 medium containing penicillin (50 U/mL) and streptomycin (50 µg/mL) supplemented with 10% WT or $TPH1^{-/-}$ serum at 37°C in a 5% CO2 atmosphere for 45 min. Acini were then stimulated in medium containing 10% mouse WT or $TPH1^{-/-}$ serum with cerulein at the concentrations indicated in the figure legends. After 15 and 60 min incubation at 37°C, the samples were fixed and stained for amylase, as described below, or assessed for amylase secretion, respectively, using the Cobas c111 analyzer (Roche). Amylase release was expressed as a percentage of secreted amylase normalized by protein content.

In vitro amylase secretion assay

105 rat pancreatic AR42J cells (ATCC, CRL-1492) cells were seeded in a 12 well plate, incubated with 50 nM dexamethasone for 24 hours to induce differentiation, then treated with the compounds at the concentrations and time indicated in the figure legend and stimulated with 10 nM cerulein. After 60 min, the acinar suspension was centrifuged and the supernatant and lysed cell pellets assayed for amylase activity.

Histology and immunohistochemistry

Detailed protocols and primary antibodies used in this study are listed in Supplementary Materials

and Methods. Quantification of TUNEL, HMGB1, Ki-67, PU.1, F4/80, CD3, HO-1-positive cells was performed in at least ten randomly selected high-power fields (×100) per slide. Non-acinar tissue areas (islets, vessels, fibrotic tissue) were excluded from the analysis.

Transcript analysis

Total RNA was extracted from pancreata as previously described.[21] Transcript levels were normalized using 18S RNA as a reference and expressed as fold induction relative to the value of untreated control animals, set as one, as described.[22] Taqman probes (Applied Biosystems) used in this study are listed in Supplementary Materials and Methods.

Results

Reduced availability of 5-HT inhibits zymogen granule secretion

To test whether 5-HT regulates secretory processes in pancreatic acinar cells, we isolated acini from wild type (WT) and TPH1$^{-/-}$ animals. Serum (Fig. S1) and pancreatic levels of 5-HT in these animals are below 10% of the WT mice, while pancreas mass and morphology are indistinguishable between the two strains.[15] TPH1$^{-/-}$ pancreatic acini accumulated amylase positive vesicles (Fig. 1A) and showed increased amylase content (Fig. 1B) compared with WT acini. In addition, while basal amylase secretion was comparable in the two strains, TPH1$^{-/-}$ acini secreted less amylase when treated with the secretagogue cerulein (Fig. 1C), suggesting that 5-HT regulates zymogen secretion in pancreatic acinar cells. To further dissect the role of 5-HT in this process, we treated dexamethasone-differentiated AR42J cells, the most commonly used cell line that maintains the secretion characteristics of normal pancreatic acinar cells,[23] with modulators of 5-HT-dependent pathways. Treatment with the 5-HT reuptake transporter (SERT) inhibitor fluoxetine, which lowers the intracellular 5-HT content, reduced both basal and cerulein-stimulated amylase secretion (Fig. 1D), without decreasing the metabolic activity of the cells (Fig. S2). Confocal analysis showed that, as observed in acini from TPH1$^{-/-}$ mice, fluoxetine treatment induced intracellular accumulation of amylase positive vesicles (Fig. 1E), indicating that the observed decrease in amylase secretion is due to impaired trafficking and not to decreased enzyme synthesis. In addition, treatment with cysteamine, a transglutaminase inhibitor which reduces the process of protein serotonylation and serotonylation–dependent secretion, [15] moderately reduced amylase secretion in AR42J cells (Fig. 1D). Conversely, amylase secretion did not change following incubation with the 5-HT2 receptor agonist ▪-methyl-5-HT, but decreased following treatment with GR127935 and LY266097, specific antagonists of the 5-HT receptors 5-HT1B and 5-HT2B expressed in mouse pancreata (data not shown and [24]) (Fig. 1D). However, both compounds also decreased the metabolic activity of the cells (data not shown), thus it is possible that the reduced secretion is secondary to impaired cellular viability.

Reduced availability of 5-HT modulates actin cytoskeleton dynamics

In vivo administration of high doses of cerulein perturbs the apical localization of zymogen granules and redirects their exocytosis to the basolateral membranes of pancreatic acinar cells. Importantly, this aberrant secretion is accompanied by reorganization of actin cytoskeleton and loss of cell polarity.[25] To analyze whether 5-HT modulates granule dynamics in vivo, we treated WT and TPH1$^{-/-}$ mice with high doses of cerulein. Zymogen granule content increased in both strains 24h after treatment, indicating that TPH1$^{-/-}$ acinar cells are responsive to the administered cerulein. As expected, in WT mice the granule polarization was perturbed, as demonstrated by the appearance of granules surrounding the nuclei and in close contact with the basolateral membranes (Fig. 2A). However, in TPH1$^{-/-}$ animals the granules maintained an apical localization and their re-localization to the basolateral membrane was rarely observed. As zymogen secretion intersects with the apical actin cytoskeleton, we tested whether the secretory defect observed in the absence of 5-HT was accompanied by cytoskeletal alterations. Staining for Rac1, a member of the Rho family involved in actin reorganization and amylase secretion in pancreatic acinar cells, [5, 6] revealed homogeneous cytosolic staining in pancreatic cryosections of untreated mice but a more pronounced re-localization to acinar periphery in TPH1$^{-/-}$ mice 8h after starting the cerulein treatment (Fig. 2B). In addition, phalloidin staining in untreated WT mice showed actin labeling predominantly in centroacinar regions and re-localization to basolateral membranes 8h after treatment, as observed in treated isolated acini.[5, 26] (Fig. 2C, arrows). Conversely, in untreated TPH1$^{-/-}$ actin labeling was clearly detected surrounding individual acinar cells, becoming more diffuse and cytosolic during cerulein stimulation. To further test the involvement of 5-HT in actin cytoskeleton dynamics we monitored the formation of cerulein-induced protrusions in acinar cells, a process dependent on actin re-organization.[5, 27] While 15 min cerulein treatment induced the typical protrusions in AR42J cells, fluoxetine pre-treatment prevented protrusion formation (Fig. 2D). Furthermore, fluoxetine also inhibited the protrusions induced by the F-actin stabilizing agent

jasplakinolide (Fig. 2D), indicating that the 5-HT-mediated effect on cytoskeleton is not dependent on cerulein stimulation. Collectively, these results suggest that 5-HT is involved in actin cytoskeleton dynamics.

Reduced availability of 5-HT limits zymogen release and leukocyte infiltration in cerulein-induced acute pancreatitis

Cerulein-induced aberrant acinar secretion, which is currently the best model for clinical interstitial AP,[28] releases zymogens into the interstitial space and eventually in the blood circulation. Thus we evaluated whether the reduced acinar cell secretion observed in absence of 5-HT ex vivo resulted in reduced zymogen release in circulation in vivo. Serum amylase (Fig. 3A) and lipase (data not shown) levels were lower in TPH1$^{-/-}$ than in WT mice at 8h and 24h after AP induction, suggesting that absence of 5-HT did indeed reduce cerulein-induced aberrant secretion. To further evaluate whether the absence of 5-HT limited leukocyte infiltration, a critical step in the pathophysiology of the disease, we analyzed pancreata 24h after AP induction when the inflammatory response is robust in the organ. Immunostaining analyses with the pan-leukocyte marker coronin-1 showed that fewer leukocytes were recruited to the pancreas (Fig. 3B) and spleen (Fig. S3) and entered the cell cycle (Fig. 3C) in TPH1$^{-/-}$ than WT mice. In parallel with the limited leukocyte infiltration, pancreatic acinar cells were less sensitive to induction of apoptosis in TPH1$^{-/-}$ than WT animals while acinar necrosis was comparable in both strains (data not shown), suggesting that 5-HT does not promote necrosis under these experimental conditions. In addition, oxidative stress, measured by lipid peroxidation levels, was not detectable (Fig. S4).

In addition, we assessed whether leukocyte subpopulations were differentially recruited in the absence of 5-HT. Immunolabeling for PU.1, expressed in myeloid cells including neutrophils and macrophages, showed fewer positive cells in TPH1$^{-/-}$ pancreata (Fig. 3D). Both staining (Fig. 3D) and mRNA quantification (data not shown) of F4/80 revealed that the amount of mature tissue macrophages did not differ significantly in the two mouse strains while neutrophil MPO levels were lower in TPH1$^{-/-}$ pancreata compared with WT mice (Fig. 3E), suggesting that 5-HT regulates this cell population in our AP model. Furthermore, staining for T-lymphocytes (CD3) and macrophage-dependent hemeoxigenase-1 (HO-1) showed a low level of infiltration comparable in the two mouse strains (Fig. 3D). This indicates not only that T-lymphocytes constitute a minor proportion of infiltrating cells but also that the induction of HO-1 in macrophages as a defense mechanism against oxidative stress is not pronounced at this time point.

Moreover, analysis of cytokine/chemokine expression showed that transcript levels of the potent chemokine MCP-1 strongly increased in WT animals after AP induction while the increase was modest in TPH1$^{-/-}$ mice, thus correlating with the low level of infiltration detected in these animals (Fig. 3F). On the contrary, the up-regulation of pro-inflammatory cytokines IL-6 and TNF-α was less pronounced than MCP-1 and comparable in the two strains, while levels of IL-1β, which is thought to contribute to the systemic effects of pancreatitis,[29] were higher in TPH1$^{-/-}$ mice upon AP induction.

Importantly, restoration of 5-HT levels in TPH1$^{-/-}$ mice via administration of the 5-HT precursor 5-HTP, as previously described,[15] induced a robust leukocyte infiltration (Fig. S5A), increased MCP-1 transcript levels (Fig. S5B) and promoted apoptosis of acinar cells (Fig. S5C) following 24h of cerulein treatment. These results confirmed that 5-HT is a main factor promoting the recruitment of inflammatory cells and cellular damage in the pancreas following AP induction.

Reduced availability of 5-HT exacerbates inflammatory and fibrotic processes during progression of pancreatitis.

Next, we evaluated whether TPH1$^{-/-}$ mice maintained a reduced inflammatory response also in the progression toward a chronic stage of the disease. Surprisingly, leukocyte infiltration was higher in TPH1$^{-/-}$ animals compared with WT mice after two weeks of cerulein treatment (Fig. 4A), but no lethality was observed throughout the course of the experiment. Time-course analysis of chemokine/cytokine expression in the pancreas revealed that in WT animals all the mRNA tested peaked either at 24h (MCP-1, Fig. 3F) or three days (IL-6, Cox-2, IL-1β) after the beginning of cerulein treatment, and progressively decreased over time (Fig. 4B). On the other hand, in TPH1$^{-/-}$ mice the expression of MCP-1, IL-6, Cox-2 (Fig. 4B) and macrophage F4/80 transcripts (Fig. 4C) were up-regulated after two weeks of treatment, when leukocyte infiltration increased. In addition, IL-1β levels, expressed also by acinar cells (Fig. S6), remained constantly higher in TPH1$^{-/-}$ animals during the entire time course of experimental pancreatitis (Fig. 4B). Moreover, cellular damage assessed by histological score was not reduced (Fig. S7), suggesting that the 5-HT-mediated protection observed during AP may be transient.

Finally, we evaluated whether the absence of peripheral 5-HT modulated the development of fibrosis in the pancreas, a process mediated by activated pancreatic stellate cells. Similar to the observations of pro-inflammatory genes, expression of TGF-β, one of the most potent fibrogenic stimuli described for pancreatic stellate

cells, [30] increased in WT mice in the early phase of pancreatitis induction and decreased after two weeks of cerulein treatment (Fig. 5). On the contrary, TPH1$^{-/-}$ mice showed increased TGF-β, α-smooth muscle actin (αSMA) and collagen transcripts at the later time point, suggesting increased induction of fibrosis in these animals.

Interestingly, administration of the 5-HT precursor 5-HTP for two weeks did not trigger inflammation when administered alone but worsened the inflammatory and fibrotic processes during cerulein treatment in WT mice to levels observed in TPH1$^{-/-}$ animals (Fig. S8). These results not only support the pro-inflammatory role of 5-HT, but also reveal the fine balance between circulating 5-HT levels and 5-HT-dependent modulation of physiopathological effects.

Reduced availability of 5-HT promotes acinar cell damage upon supramaximal cerulein stimulation.

As lack of peripheral 5-HT was associated with more severe progression of pancreatitis, we evaluated whether the observed impaired zymogen secretion led to intracellular zymogen accumulation and increased cytotoxicity following prolonged cerulein treatment. Acinar cells of TPH1$^{-/-}$ mice showed increased amylase levels (Fig. 6A) and necrosis (Fig. 6B) following two weeks cerulein treatment compared with WT animals. In addition, trypsinogen activation, an event mediating the initial pancreatic injury but not inflammation following supramaximal cerulein stimulation,[31] was observed earlier in TPH1$^{-/-}$ mice during the acute phase of experimental pancreatitis (Fig. 6C). To further test whether intracellular zymogen accumulation increases the sensitivity of acinar cells to cerulein-mediated toxicity, we treated dexamethasone-differentiated AR42J cells first with fluoxetine for 24h and then with supra- and submaximal cerulein concentrations for additional 24h. In support of our hypothesis, we found that fluoxetine treatment decreased AR42J viability only in presence of supramaximal cerulein concentration (Fig.6D).

Discussion

The role of 5-HT and 5-HT-dependent pathways outside the central nervous system is now the subject of increasing interest, as recent studies highlighted its involvement in the physiology and pathology of several organs [32]. In this study we explored the role of 5-HT in pancreas physiology and during development of pancreatitis, a highly debilitating and potentially lethal disease whose pathophysiology remains undefined. Our work using transgenic mice genetically deficient in peripheral 5-HT revealed a 5-HT-mediated modulation of zymogen secretion in pancreatic acinar cells and demonstrated a dual role of 5-HT in the onset and establishment of pancreatitis.

5-HT and the onset of pancreatitis

Pancreatic damage following deregulated zymogen secretion and leukocyte infiltration are characteristic early events in acute pancreatitis.[3, 33] In the present study we observed that both parameters markedly decreased in TPH1$^{-/-}$ mice and were restored following 5-HTP supplementation, indicating that peripheral 5-HT is a potent activator of tissue damage and inflammatory response at the onset of pancreatitis.

Early studies using pharmacological modulation of 5-HT pathways in rodents suggested that 5-HT may be involved in the development of pancreatitis. Specifically, lowering the endogenous 5-HT levels by temporarily inhibiting 5-HT synthesis or following depletion of platelets, the major store of circulating 5-HT, reduced the damage and inflammation of experimental acute pancreatitis.[34, 35] Moreover, administration of selective agonists and antagonists of different 5-HT receptors showed aggravating and protective effects, respectively, in the context of both acute and chronic pancreatitis, albeit discrepancies resulting from varying binding affinities and potentially undefined side effects of the used compounds were observed.[34, 36, 37, 38, 39]

While these studies supported the hypothesis that 5-HT exacerbates pancreatitis via specific receptor-mediated pathways, the exact mechanisms of 5-HT modulation of the disease were not identified. As 5-HT has been shown to directly activate and induce the production of pro-inflammatory cytokines in isolated immune cells (reviewed in [40, 41]), it is likely that the absence of peripheral 5-HT directly hampered leukocyte activation also in our experimental model. However, it is possible that 5-HT affected the production of immune-mediators also in non-leukocytic cells, including pancreatic acinar cells. Indeed, the CC chemokine MCP-1, which showed the highest up-regulation in WT animals during pancreatitis and only moderate transcription in the absence of 5-HT, is produced also by acinar cells (reviewed in [42]). The critical role of MCP-1 as an early inflammatory mediator driving leukocyte chemotaxis into the injured pancreas was revealed by Bhatia and colleagues.[43] Importantly, despite the fact that MCP-1 is regarded as an activator of monocytes, recent studies suggested that this chemokine is also promoting the recruitment of neutrophils during the course of pancreatitis and colitis,[43, 44] implicating a wide range of target cells responsive to the molecule.

The pattern of pro-inflammatory processes we observed in our AP model suggested that the

reduction of pancreatic inflammation in TPH1$^{-/-}$ mice cannot be attributed to a general reduction of pro-inflammatory factors, but likely to an aberrant regulation of selected chemokines/cytokines. This complex scenario parallels the broad variety of 5-HT-mediated effects, largely due to the presence of multiple receptor subtypes in different cell types. In particular, 5-HT was shown to display opposite effects on the production of cytokines with similar cellular functions (reviewed in [13, 41]). Of note, while 5-HT receptors have been characterized on the surface of peripheral lymphocytes, macrophages and dendritic cells, the expression of these receptors on neutrophils has not yet been described,[11, 40] suggesting that 5-HT modulation of these cells, as previously observed in a murine model of experimental colitis,[12] may be 5-HT receptor-independent and/or driven by other immune-modulators.

In addition, 5-HT is likely to influence pancreatic inflammation via its vasoactive properties. In this regard, increased serum levels of 5-HT metabolites were detected following AP.[37] These metabolites, which are potent activators of platelet aggregation, vasoconstriction and reduced tissue microcirculation, are thought to impair pancreatic microcirculation, which is recognized as one of the etiological events of AP.[45, 46] Conversely, absence of 5-HT would maintain normal microcirculation properties during inflammatory injuries, as it was recently shown in TPH1$^{-/-}$ animals during challenge with experimental hepatitis.[47]

Finally, a novel receptor-independent mechanism identified in our study by which 5-HT could modulate the development of pancreatitis is the regulation of cellular secretion. We showed both in vitro and in vivo that reduced availability of 5-HT modulated actin cytoskeleton dynamics prevented the aberrant re-localization of zymogen granules and decreased amylase secretion, potentially limiting the initial magnitude of the immunoresponse. In addition to the observed reduction of zymogen secretion, it is tempting to speculate that absence of 5-HT may also reduce the efficiency of cytokine/chemokine secretion both in leukocytes and acinar cells, thus contributing to the limited inflammation observed in TPH1$^{-/-}$ animals. Moreover, an additional factor that could contribute to the differential development of AP and warrant of further investigation is the possible alteration in ductal secretion, as 5-HT was shown to inhibit fluid secretion in guinea pigs, albeit at supraphysiological concentration. [48]

As previously mentioned, TPH1$^{-/-}$ animals have impaired insulin secretion by β-cells and develop mild diabetes which worsens during aging (70 weeks), but neither reduces life expectancy nor causes morbidity.[15] Currently, we do not have evidences for a role of diabetes in the development of pancreatitis observed in TPH1$^{-/-}$ mice. Indeed, while diabetes has been shown to aggravate cerulein-induced pancreatitis by increasing inflammation during the acute phase and promoting metaplasia,[49] these events were not observed in our experimental model.

We are currently focusing on identifying the molecular targets of 5-HT controlling acinar cell secretion. Our results demonstrating that intracellular 5-HT is required for zymogen exocytosis suggested that serotonylation of intracellular proteins is required for optimal zymogen secretion, as previously observed for serotonylated small GTPases of the Rab family in pancreatic β-cells and platelets.[14, 15] While Rab GTPases, central regulators of vesicle budding, motility and fusion in eukaryotic cells, are likely to be involved in acinar cells zymogen secretion, our results showing 5-HT modulation of actin–based processes suggest that serotonylation of proteins regulating cellular cytoskeleton dynamics, including members of the Rho family, may contribute as well to zymogen secretion in acinar cells.

5-HT and progression of pancreatitis

Our AP data and a recent study on the pathogenesis of experimental colitis,[12] highlight a possible general involvement of 5-HT in the development of inflammatory diseases and support the therapeutic potential of agents interfering with 5-HT-mediated pathways. However, when we monitored the progression of pancreatitis toward a chronic state, we found that the blunted response in the absence of 5-HT was transient and exacerbated in TPH1$^{-/-}$ mice after two weeks of cerulein treatment. Hence, these results indicated that the immune response does not completely rely on 5-HT-mediated mechanisms, but it is likely to activate compensatory mechanisms at later stages of the disease.

The molecular mechanisms leading to the increased cellular damage in TPH1$^{-/-}$ mice after two weeks of cerulein treatment remain to be determined. One possibility highlighted by our results is that a prolonged impairment of zymogen secretion is toxic to acinar cells. In this context, the observed higher number of necrotic acinar cells in TPH1$^{-/-}$ may constitute a trigger for a necrosis-fibrosis sequence, as described for chronic pancreatitis.[50, 51] As typical hallmarks for this process we detected macrophage recruitment and up-regulation of TGF-β expression, stellate cell activation and extracellular matrix markers in TPH1$^{-/-}$ animals, indicating an increased fibrogenesis in our experimental system.

Alternatively, increased cellular damage could derive from impaired regeneration of acinar cells in

absence of 5-HT, as previously observed in the case of hepatocytes following experimental hepatectomy.[8] Indeed, we observed that cell cycle activation in acinar cells was altered in TPH1$^{-/-}$ mice following cerulein-induced pancreatitis (Saponara et al., in preparation).

In conclusion, our data directly link 5-HT to the pathophysiology of cerulein-induced pancreatitis and provide a mechanistic explanation for the reduced acute stage of the disease observed in absence of 5-HT. These findings underline the involvement of 5-HT in the secretory processes of pancreatic acinar cells and in the regulation of pro-inflammatory mediators and subsequent progression of inflammatory diseases. Collectively, our data not only support the potential of therapeutic strategies based on 5-HT-mediated pathways during pathological inflammatory events, but also contribute to the elucidation of cellular processes in the exocrine pancreas.

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Competing interests None

Contributors The authors of this manuscript contributed in the study design, acquisition, analysis, interpretation of data, drafting and critical revision of the manuscript.

Data sharing statement No additional data

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Figures

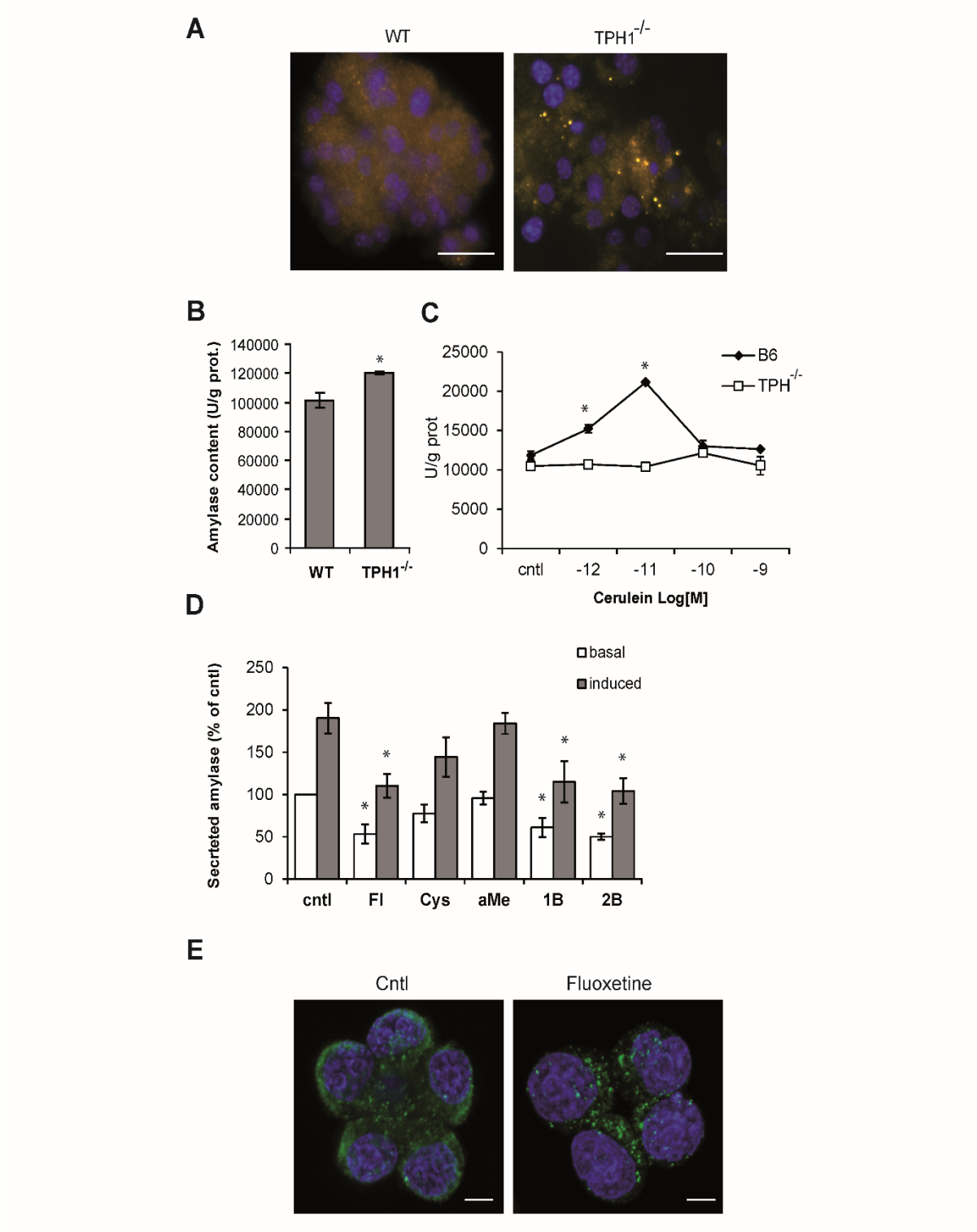

Figure 1. 5-HT modulates zymogen secretion in pancreatic acinar cells. A. Isolated acini were fixed and stained with an anti-amylase antibody (yellow). Nuclear DNA was stained with DAPI (blue). Note the accumulation of amylase containing vesicles in TPH1$^{-/-}$ acini. Scale bars: 50 µM. B. Amylase content in WT and TPH1$^{-/-}$ acini normalized by protein amount. C. Isolated acini were treated with cerulein at the indicated concentrations for 60 min and basal and cerulein-induced amylase secretion was normalized by protein amount. Note the reduced amylase secretion in stimulated TPH1$^{-/-}$ acini. D. AR42J cells were treated for 5h with 20 µM fluoxetine (Fl), 500µM cysteamine (Cys), 10 µM α-methyl-5-HT (aMe), 10 mM HTR1B antagonist GR127935 (1B), 10 µM HTR2B antagonist LY266097 (2B) or solvent (Cntl). Basal and cerulein-induced amylase secretion was expressed as percent of control-treated cells. E. Confocal microscopy of AR42J cells treated for 5h with 20 µM fluoxetine (Fl) and stained with an anti-amylase antibody (green). Nuclear DNA was stained with DAPI (blue). Note the accumulation of amylase-containing vesicles upon fluoxetine treatment. Scale bars: 10 µM. Results are average ± SEM (n=3), *p<0.05.

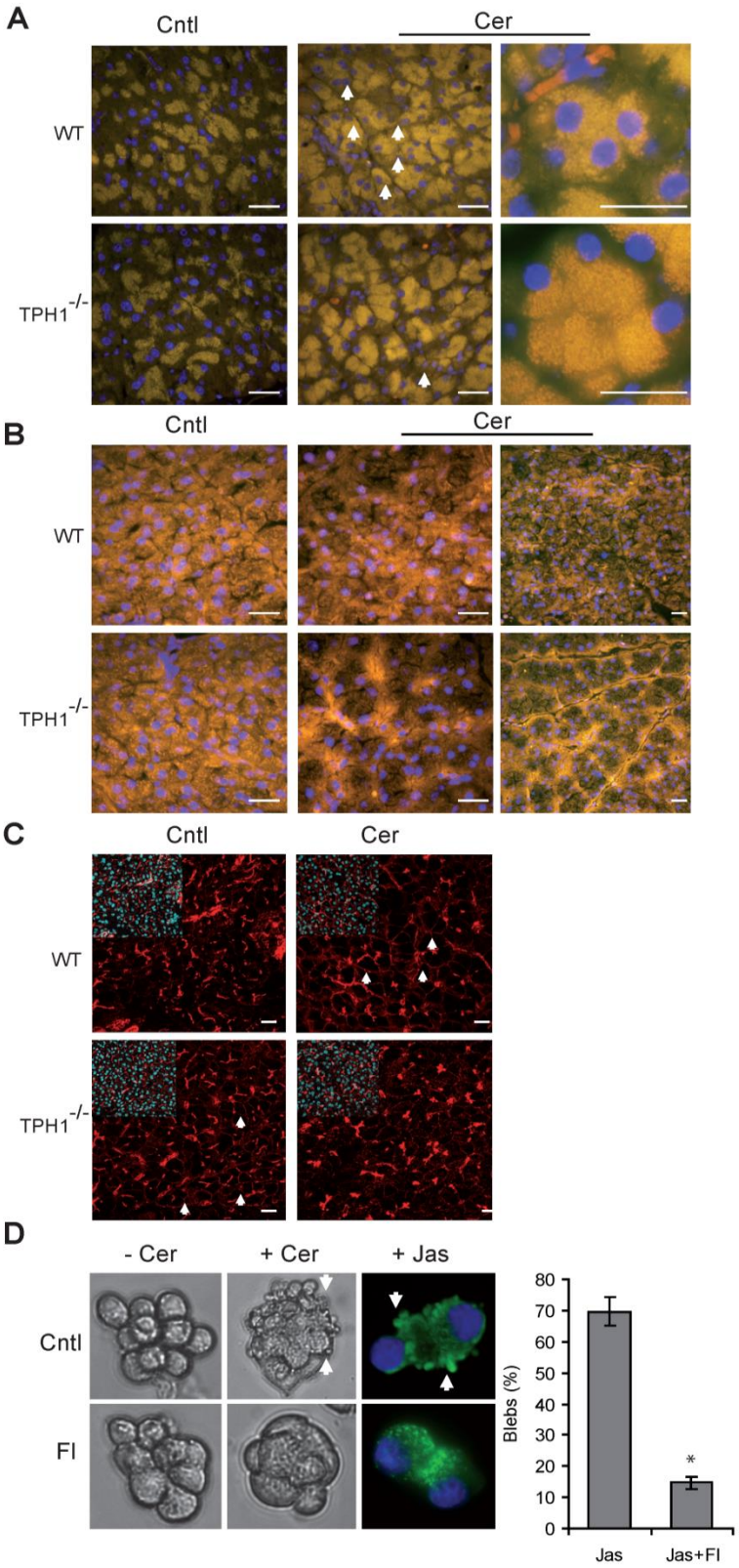

Figure 2. 5-HT modulates cytoskeleton dynamics in pancreatic acinar cells. A. Visualization of zymogen granules in control and treated mice harvested 24h after the first cerulein injection showed increased apical to baso-lateral granule re-localization in WT animals (arrows). Scale bars: 50 µM. Rac1 (B) and actin (C) staining in control and treated mice harvested 8h after the first cerulein injection. Note the different dynamic of baso-lateral staining (arrows) in absence of 5-HT. Insets, merge pictures with DAPI nuclear staining. Scale bars: 50 µM D. Left panel. Bleb formation in AR42J cells treated for 30 min with 10 nM cerulein or 1 µM jasplakinolide (Jas) was prevented by 5h pre-treatment with 20 mM fluoxetine (Fl). Right panel: enumeration of bleb forming cells following 1 µM jasplakinolide treatment (Jas) in presence or absence of 20 mM fluoxetine (Fl). Results are expressed as percentage of total cell number ± SEM (n=3), *p<0.05.

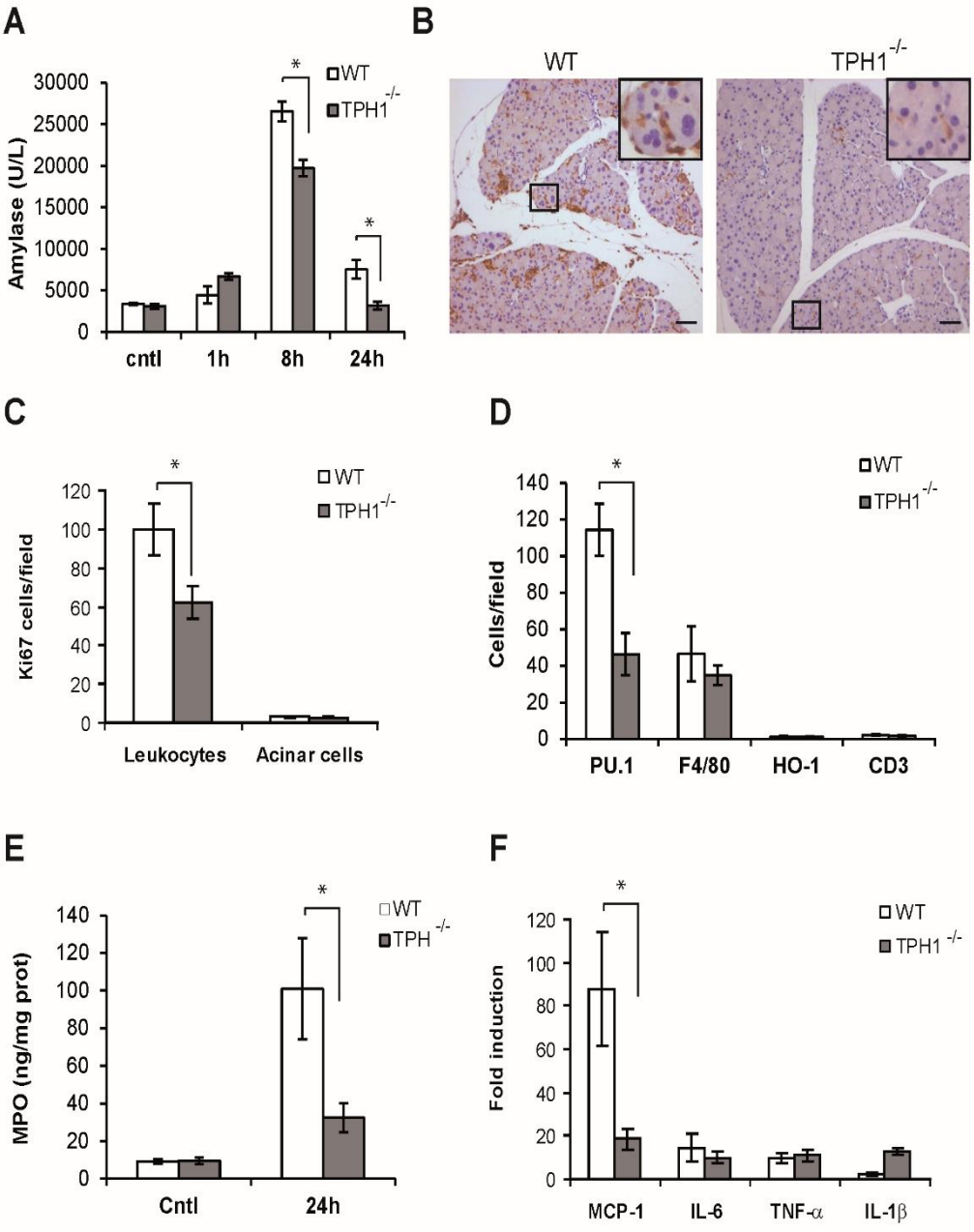

Figure 3. Lack of 5-HT limits damage and leukocyte infiltration during AP. A. Serum levels of amylase increased more in WT than TPH1$^{-/-}$ mice during cerulein treatment. B. Staining with the pan-leukocyte marker coronin-1 revealed higher leukocyte infiltration in pancreas of WT mice compared with TPH1$^{-/-}$ animals. Insets, enlarged view of infiltrating cells shown by frame. Scale bars: 50 µM. C. Quantification of Ki67 positive infiltrating and pancreatic acinar cells. D. Quantification of infiltrating cells expressing PU.1, F4/80, HO-1, CD3 in pancreas. E. Quantification of MPO levels in the pancreas. F. qRT-PCR of chemokine/cytokine levels in the pancreas. Note the limited up-regulation of MCP-1 in TPH1$^{-/-}$ animals. Results are average ± SEM (n=5), *p<0.05.

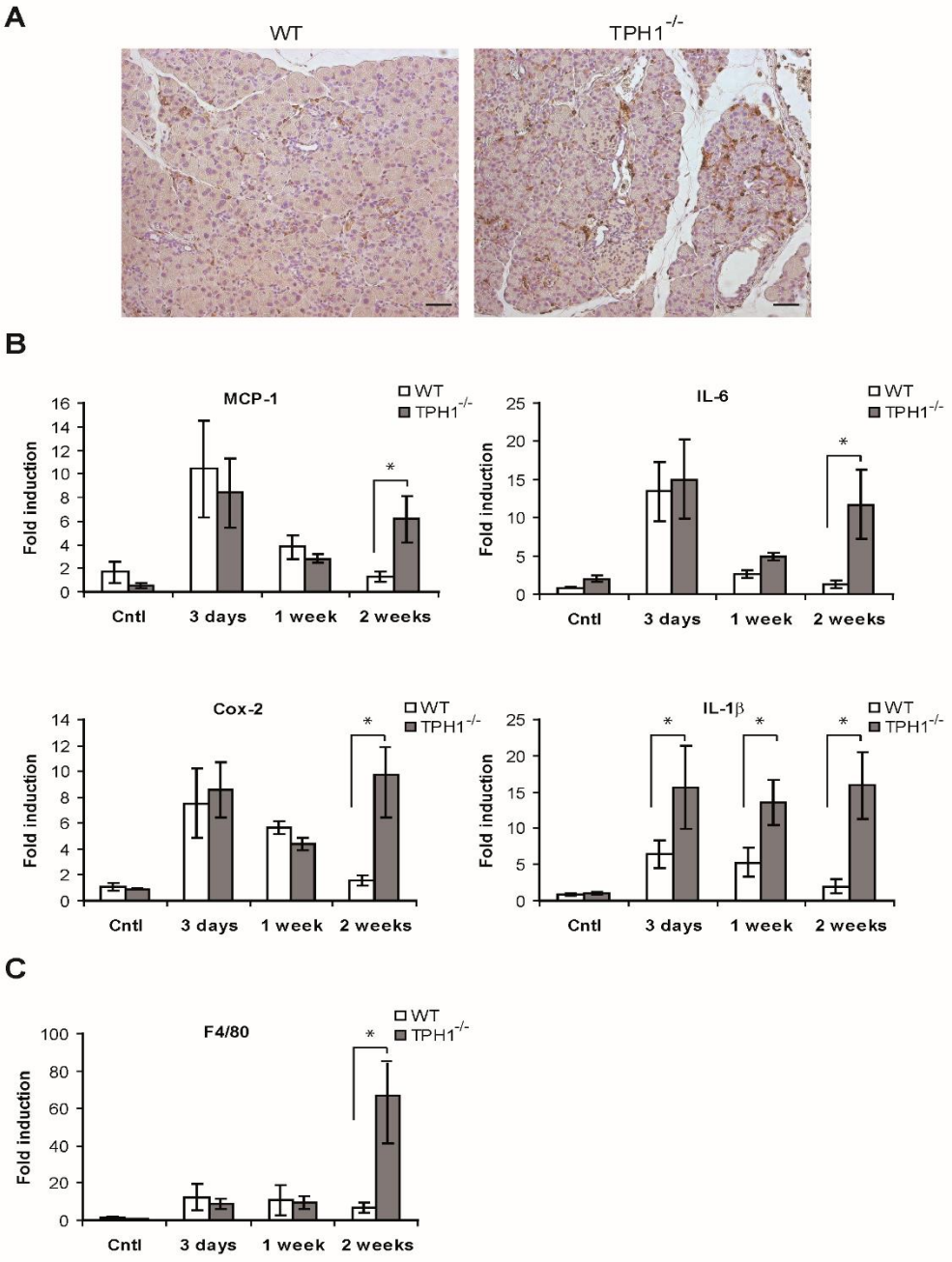

Figure 4. Lack of 5-HT exacerbates inflammatory processes after two weeks of cerulein treatment. A. Staining with the pan-leukocyte marker coronin-1 revealed higher leukocyte infiltration in the pancreas of TPH1$^{-/-}$ animals. Scale bars: 50 µM. qRT-PCR showed up-regulation of chemokine/cytokine levels (B) and macrophage-specific antigen F4/80 (C) in the pancreas of TPH1$^{-/-}$ mice after two weeks of cerulein treatment. Results are average ± SEM (n=5), *p<0.05.

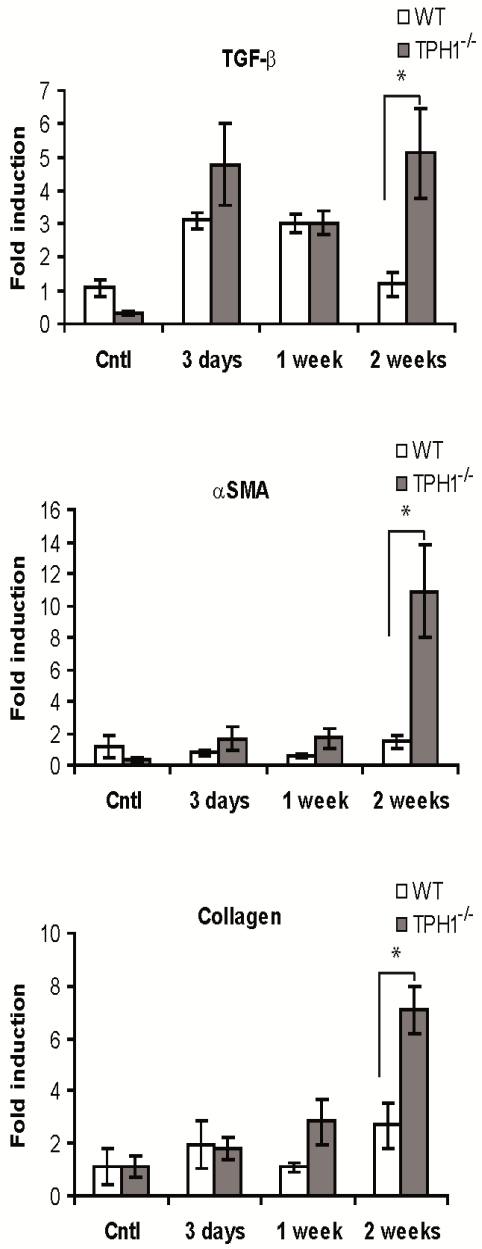

Figure 5. Lack of 5-HT exacerbates fibrotic processes after two weeks of cerulein treatment. qRT-PCR quantification of TGF-β, α-smooth muscle actin (αSMA) and collagen transcripts indicated increased activation of fibrotic processes in TPH1$^{-/-}$ mice after two weeks of cerulein treatment. Results are average ± SEM (n=5), *p<0.05.

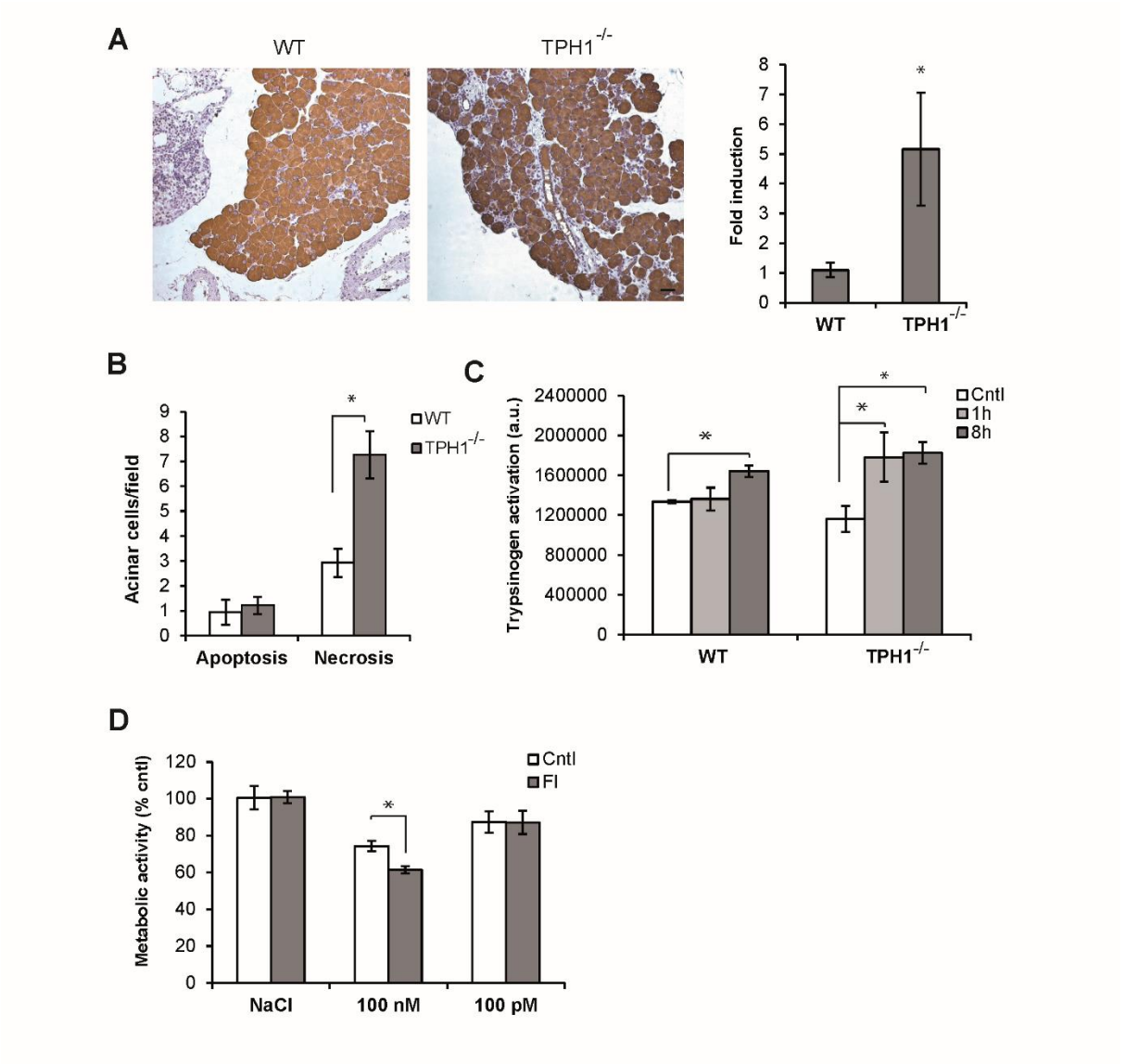

Figure 6. Lack of 5-HT promotes acinar cell damage upon supramaximal cerulein stimulation. A. Amylase immunostaining (left panel) and transcript expression (right panel) after two weeks of cerulein treatment.B. Quantification of apoptotic and necrotic pancreatic acinar cells after two weeks of cerulein treatment. C. Quantification of trypsinogen activation after one and eight hours of cerulein treatment. D. Dexamethasone-differentiated AR42J cells were treated for 24h with solvent (Cntl) or 20 µM fluoxetine (Fl) followed by supramaximal (100 nM) and submaximal (100 pM) cerulein or saline control (NaCl) for additional 24h. Their viability was assessed by measuring metabolic activity through reduction of MTT and expressed as % of control treated cells. Results are average ± SEM (n=5), *p<0.05.

Supplementary Materials and Methods

Materials and Methods

Biochemical reagents

Unless otherwise stated, all chemicals were purchased from Sigma and cell culture reagents from Gibco-BRL. Reagents stock solutions were prepared at the following concentrations in 0.9% NaCl: 50 mM cysteamine, 0.37 mM cerulein, 5 mg/mL 5-hydroxy-L-tryptophan (5-HTP), 33 mM α-methyl-5-HT, 10 mM HTR2B (LY266097,Tocris Bioscience, Bristol, UK), 10 mM HTR1B (GR127935, Tocris Bioscience, Bristol, UK), 5.8 mM fluoxetine. 100 µM Jasplakinolide was prepared in DMSO.

Mammalian cell culture

AR42J cells were maintained in Kaighn's modified Ham's F-12 medium with 20% fetal bovine serum (FBS), 50 U/mL penicillin, and 50 µg/mL streptomycin at 37°C in a 5% CO2 atmosphere. Cell metabolic activity was tested with 0.5% tetrazolium salt solution 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), according to the manufacturer's instructions.

Biochemical analysis of blood

For determination of pancreatic enzymes present in the serum, blood was sampled by heart puncture and amylase and lipase levels were measured using the Cobas c111 spectrophotometer (Roche Diagnostic GmbH, Mannheim, Germany). LDH levels were measured using the Fuji Dri-Chem 4000i analyzer.

5-HT assay

5-HT quantification in plasma was performed using a 5-HT ELISA kit (Griffin Life Science, Abington, UK), according to the manufacturer's instructions.

Histology and immunohistochemistry

Pancreas and spleen specimens were embedded in paraffin or OCT for histological analyses as described.[1] For immunofluorescence analysis of cultured cell lines, AR42J cells were fixed in 3.6% formaldehyde and permeabilized with 0.2% Triton X-100 in PBS. Primary antibodies used in this study were: Alexa Fluor 594 phalloidin (Life Technologies, Zug, Switzerland), rabbit anti-amylase (Sigma-Aldrich, Buchs, Switzerland), mouse anti-Rac1, rabbit anti-IL-1β, rat anti-F4/80, rabbit anti-HMGB-1 (Abcam, Cambridge, UK), rabbit anti-PU.1 (Cell Signaling Technologies, Danvers, MA), rabbit anti-coronin-1 [2], rabbit anti-Ki67 (Abcam, Cambridge, UK), rabbit anti-CD3 and rabbit anti-HO-1 (Assay Designs, Ann Arbor, USA). Fluorophore-conjugated secondary antibodies were used at 1:200 dilution. Nuclei were visualized with 4′, 6-diamidino-2-phenylindole (DAPI).

Detection of DNA fragmentation in apoptotic cells was performed with a TUNEL assay using an ApopTag peroxidase Kit (MP Biomedicals, Illkirch, France).

Microscopy analyses were performed on a Zeiss Axioplan 2 Imaging fluorescence microscope (Carl Zeiss Microimaging, Göttingen, Germany) or on a Leica SP2 AOBS confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany), using the appropriate settings. Image stacks of optical sections were further processed using the Huygens deconvolution software package version 2.7 (Scientific Volume Imaging, Hilversum, NL).

MPO assay

10 mG frozen pancreatic tissue was homogenized in 200 µL cold lysis buffer (200 mM NaCl, 5 mM EDTA, 10 mM Tris, 10% glycerol, 1mM PMSF, complete protease inhibitor, pH 7.4). Myeloperoxidase activity in the supernatant was measured using the Mouse MPO ELISA Kit (HBT, Hycult Biotech,

Uden, The Netherlands), according to the manufacturer's instructions. Measured MPO levels were normalized by protein concentration.

Malondialdehyde level determination

Free malondialdehyde (MDA) levels in pancreas tissues were quantified using a MDA-586 colorimetric assay kit (Oxis International Inc., CA, USA) according to the manufacturer instructions. Briefly, frozen pancreata were homogenized in presence of butylated hydroxytoluene at a concentration of 0.1 mg/µL. Measured MDA levels were normalized by protein concentration.

Transcript analysis

The following Taqman probes (Applied Biosystems) were used: F4/80-Emr1 Mm00802530_m1, MCP1/CCL2 Mm00441242_m1, IL-6 Mm00446190_m1, IL-1β Mm00434228_m1, TNFα Mm00443258_m1, COX-2 Mm00478374_m1, TGFβ Mm00441724_m1, αSMA Mm01546133_m1, collagen type IV, α1 Mm01210125_m1, amylase 1 Mm00651524_m1.

Trypsin activity assay

20 mG frozen pancreatic tissue was homogenized in 250 µL cold MOPS buffer (250 mM sucrose, 5 mM MOPS, 1 mM Mg SO4, pH 6.5) using the Precellys® 24 tissue homogenizer. Trypsin activity was measured flourometrically at 37°C in 250 µL trypsin assay buffer (50 mM Tris, 150 mM NaCl, 1 mM CaCl2, 0.1% BSA, pH 8.0) containing 250 µg supernatants and 40 µM Boc-Glu-Ala-Arg-MCA (Bachem, Bubendorf, Switzerland) as a substrate, as described [3]. The fluorescence emitted at 440 nm in response to excitation at 380 nm was monitored over a 10 min period.

Statistical analyses

Groups of 5 animals were tested for each experiment. The data are expressed as the means ± SEM. The statistical significance of differences in the means of experimental groups was determined using an unpaired, two-tailed Student's t test or one-way analysis of variance (GraphPad Prism 4.0c; GraphPad Software, Inc.), and a probability value <0.05 was considered statistically significant. When the overall probability value was <0.05, the Dunnett multiple-comparisons test was used as a post-test to determine whether there was a significant difference between values of control (reference sample) and samples of interest.

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Figures

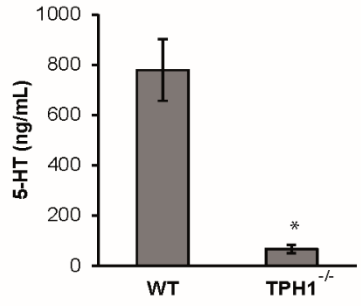

Figure S1. Content of 5-HT in platelet-rich plasma in WT and TPH1$^{-/-}$ mice. Results are average ± SEM (n=3), *p<0.05.

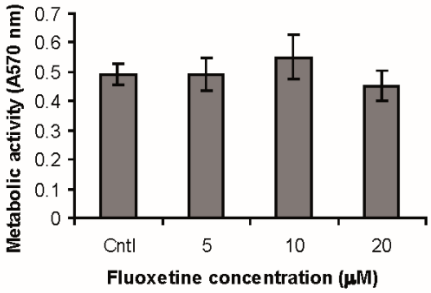

Figure S2. Fluoxetine does not reduce AR42J cell viability. AR42J cells were treated for 48h with solvent (Cntl) or fluoxetine (Fl) at the indicated concentrations and their viability was assessed by measuring metabolic activity through MTT reduction. Results are average ± SEM (n=4).

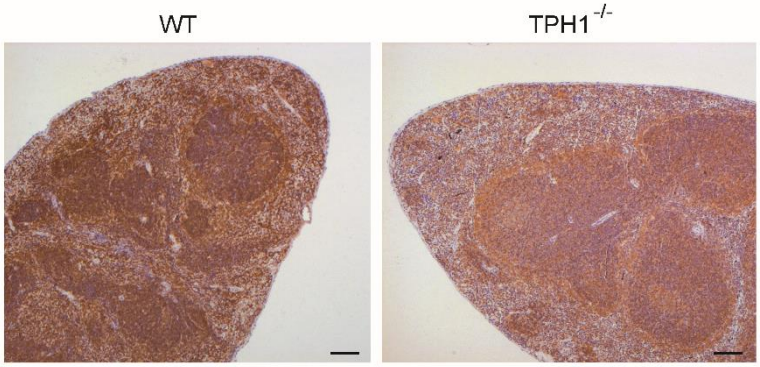

Figure S3. Lack of 5-HT limits leukocyte infiltration during cerulein-induced AP. WT and TPH1$^{-/-}$ mice were harvested 24h after the first cerulein injection. Staining with the pan-leukocyte marker coronin-1 revealed higher leukocyte infiltration in spleen of WT mice compared with TPH1$^{-/-}$ animals. Scale bars: 50 µM.

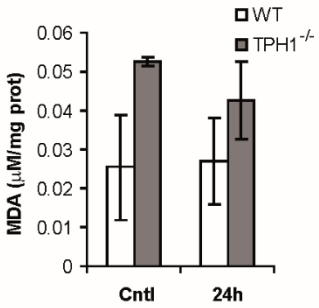

Figure S4. Lack of 5-HT does not change lipid oxidation levels. Malondialdehyde (MDA) levels were quantified in pancreatic tissue 24h after initial cerulein injection in WT and TPH1$^{-/-}$ mice using a MDA-586 colorimetric assay kit (Oxis International Inc., CA, USA) and normalized by protein concentration. Results are average ± SEM (n=5).

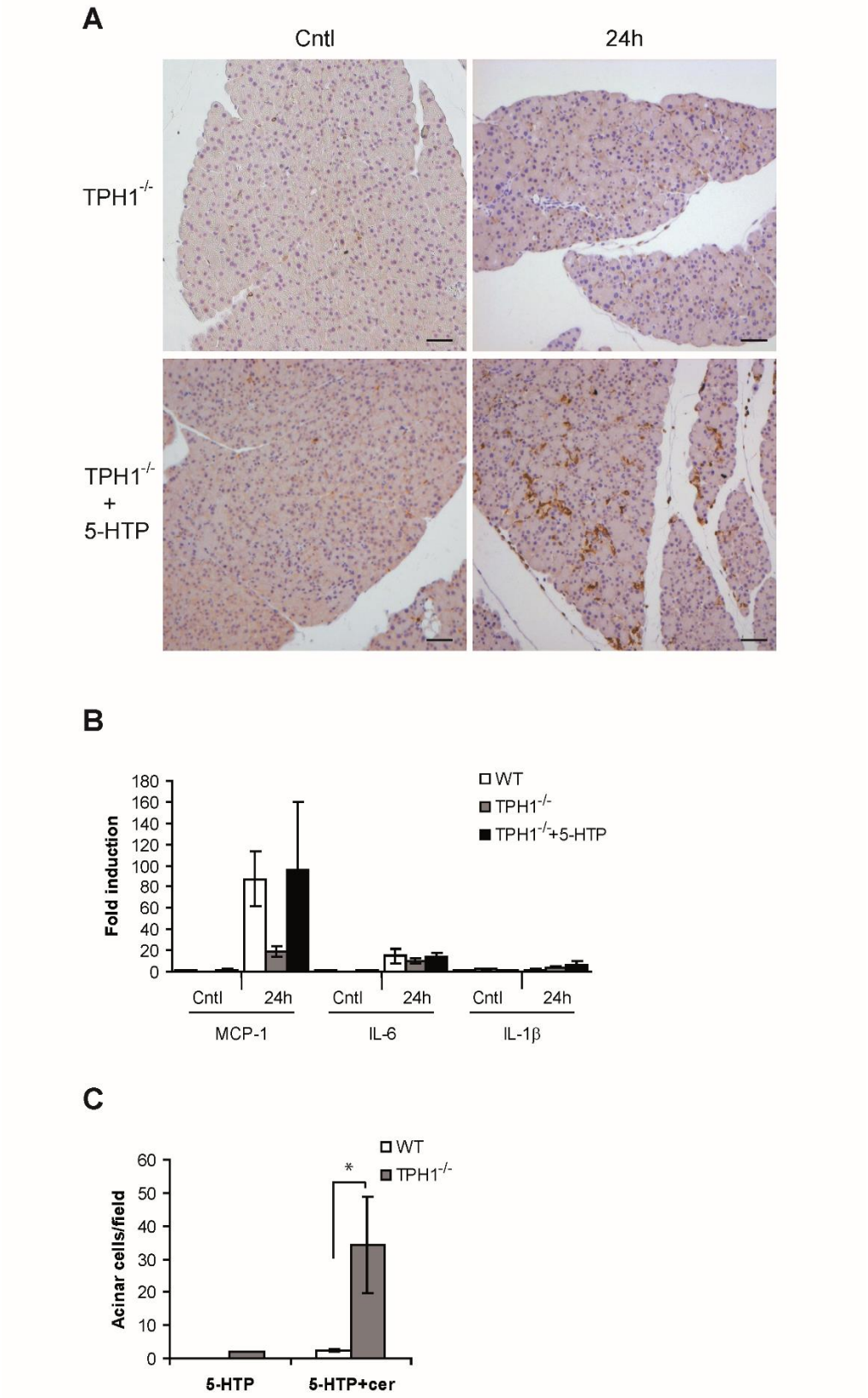

Figure S5. 5-HTP administration to TPH1$^{-/-}$ mice restores leukocyte infiltration during AP. A. Staining with the pan-leukocyte marker coronin-1 showed increased leukocyte infiltration in the pancreas upon 5-HTP treatment. Scale bars: 50 µM. B. qRT-PCR of chemokine/cytokine levels in the pancreas after 5-HTP supplementation. Values of control and cerulein-treated WT and TPH1$^{-/-}$ mice are shown as comparison. Results are average ± SEM (n=5).C. TUNEL staining showed increased number of apoptotic acinar cells in 5-HTP-treated TPH1$^{-/-}$ mice compared with WT animals following cerulein injections (5-HTP+cer). Results are average ± SEM (n=5), *p<0.05.

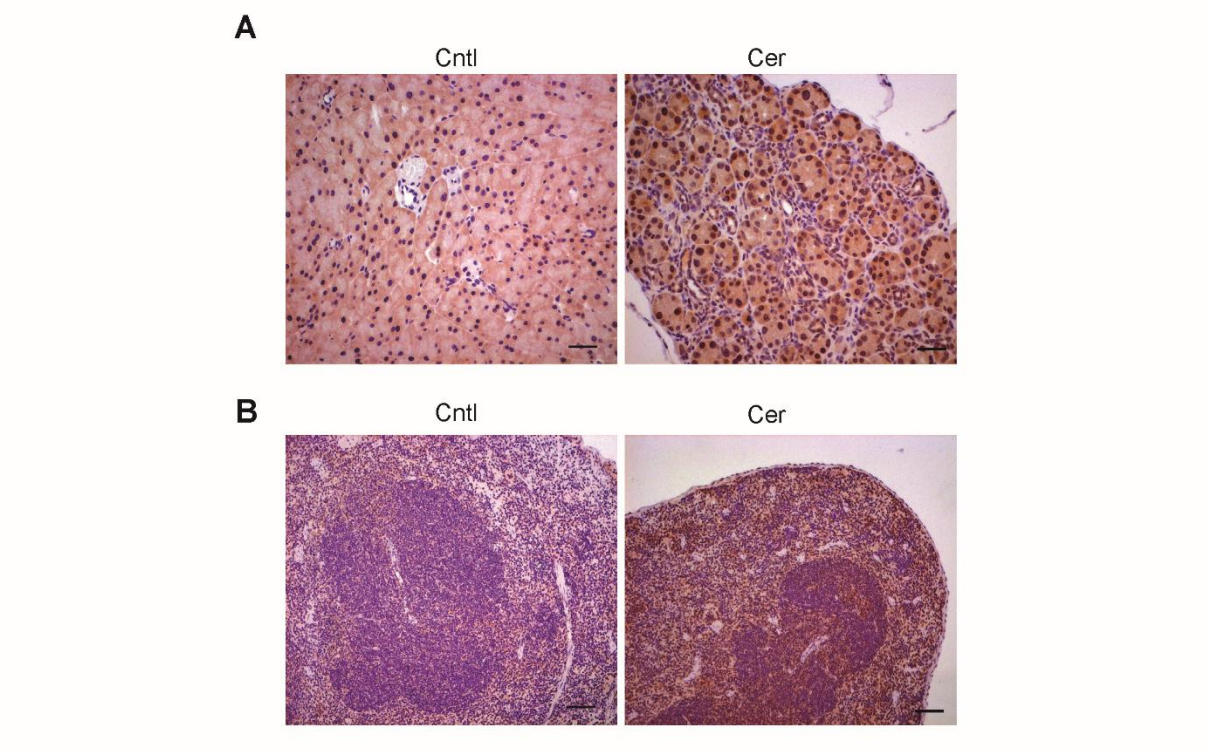

Figure S6. Cerulein treatment increase IL-1β production in acinar cells. TPH1$^{-/-}$ mice were injected repetitively with cerulein and harvested two weeks after the first injection. IL-1β staining showed increased cytokine content in acinar cells (A) and spleen (B).

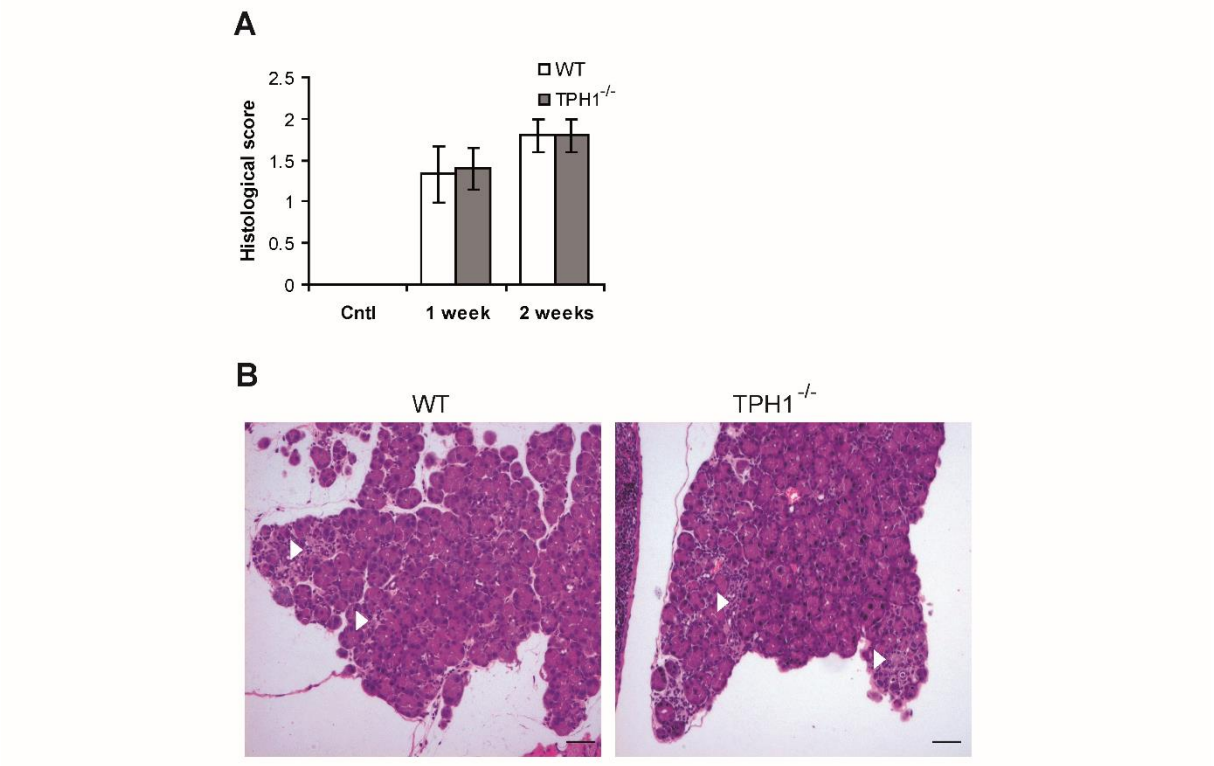

Figure S7. Lack of 5-HT does not limit cellular damage during cerulein-induced CP. WT and TPH1$^{-/-}$ mice were injected repetitively over two weeks with cerulein and harvested one and two weeks after the first injection. A. Histological score showing comparable levels of tissue damage in the two animal strains. B. Hematoxylin-eosin (H&E)-stained sections of pancreas after two weeks of cerulein-treatment. Arrowheads indicate regions of tissue damage. Scale bars: 50 µM

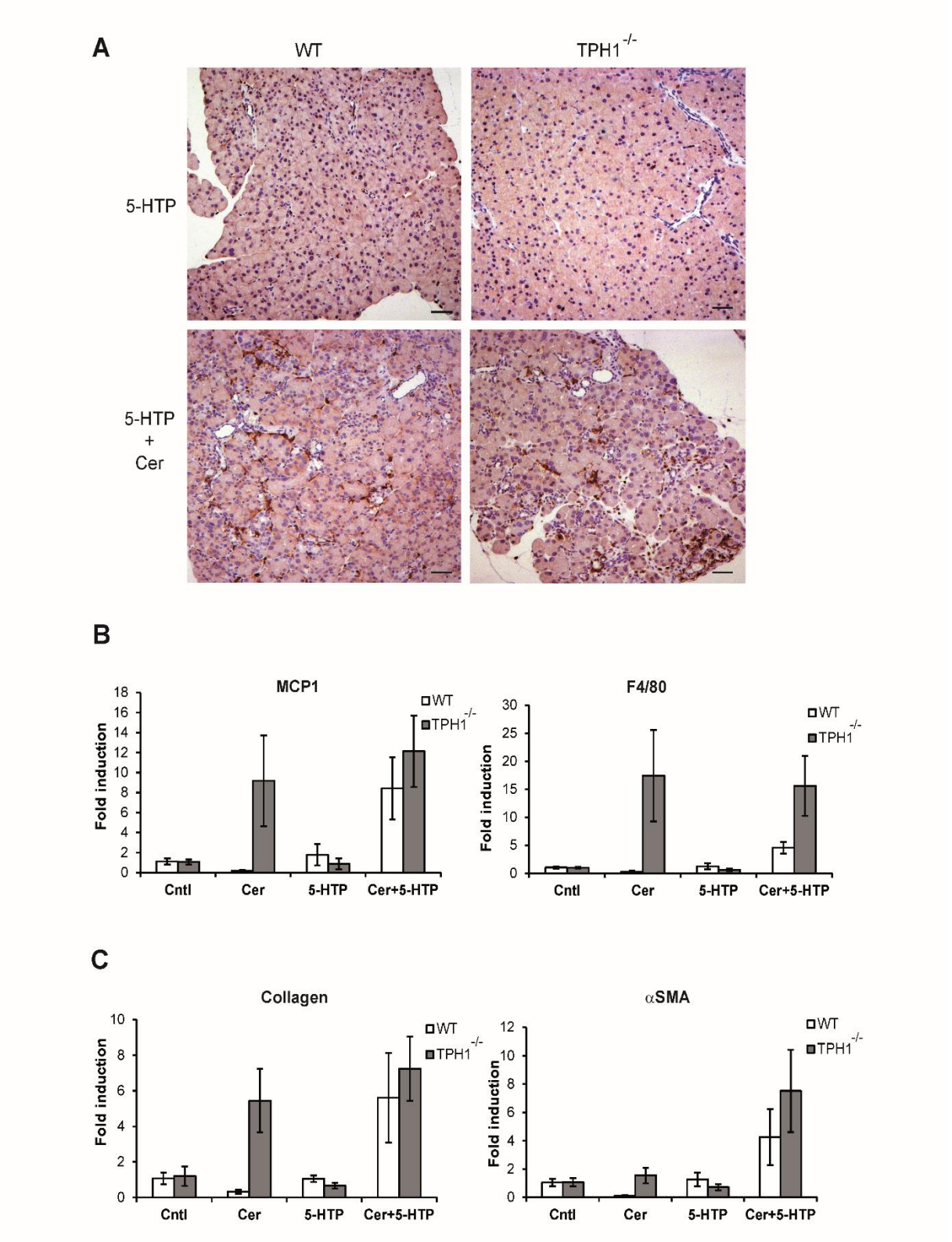

Figure S8. 5-HTP administration increases inflammatory and fibrotic processes during two weeks cerulein treatment. A. Staining with the pan-leukocyte marker coronin-1 showed increased leukocyte infiltration in the pancreas of WT and TPH1$^{-/-}$ mice upon 5-HTP+cerulein treatment. Scale bars: 50 µM. qRT-PCR of chemokine/cytokine levels (B) and fibrotic markers (C) in the pancreas after two week cerulein treatment with or without 5-HTP supplementation. Values of control and cerulein-treated WT and TPH1$^{-/-}$mice are shown as comparison. Results are average ± SEM (n=5).

6. Manuscript B

Serotonin promotes acinar de-differentiation following pancreatitis-induced regeneration in the adult pancreas.

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Contribution: This study represents a major part of my PhD work. All experimental work was done by me with the exception of confocal microscopy analysis performed by Prof. A. Hehl, electron microscopy performed by E. Schraner and pancreatic microsurgery performed by Prof. Y. Tian. Minor experiments were performed in collaboration with colleagues. I also contributed to drafting/revising of large parts of the manuscript.

Serotonin promotes acinar de-differentiation following pancreatitis-induced regeneration in the adult pancreas.

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Running title: serotonin and acinar cell de-differentiation

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Abstract

The exocrine pancreas exhibits a distinctive capacity for tissue regeneration and renewal following injury. This regenerative ability has important implications for a variety of disorders, including pancreatitis and pancreatic cancer, diseases associated with high morbidity and mortality. Thus, understanding its underlying mechanisms may help develop therapeutic interventions. Serotonin has been recognized as a potent mitogen for a variety of cells and tissues. Here we investigated whether serotonin exerts a mitogenic effect in pancreatic acinar cells in three regenerative models, namely inflammatory tissue injury following pancreatitis, tissue loss following partial pancreatectomy and thyroid hormone-stimulated acinar proliferation. Genetic and pharmacological techniques were used to modulate serotonin levels *in vivo*. Acinar de-differentiation and cell cycle progression during the regenerative phase were investigated over the course of two weeks. By comparing acinar proliferation in the different murine models of regeneration, we found that serotonin did not affect clonal regeneration of mature acinar cells. However, it was required for acinar de-differentiation following inflammation-mediated tissue injury. Specifically, lack of serotonin resulted in delayed up-regulation of progenitor genes, delayed formation of acinar-to-ductal metaplasia and defective acinar cell proliferation. We identified serotonin-dependent acinar secretion as a key step in the progenitor-based regeneration, as it promoted acinar cell de-differentiation and the recruitment of type 2 macrophages. Finally, we identified a regulatory Hes1-Ptfa axis in the uninjured adult pancreas activated by zymogen secretion. Our findings indicate that serotonin plays a critical role in the regeneration of the adult pancreas following pancreatitis by promoting de-differentiation of acinar cells.

Keywords: serotonin; acinar secretion; de-differentiation; regeneration; ADM; pancreatitis; partial pancreatectomy.

Introduction

Despite a low homeostatic turnover, the adult exocrine pancreas has the ability to regenerate following an inflammatory insult. An increasing number of reports have revealed that during this regenerative process acinar cells, which constitute the most abundant cell population of the exocrine tissue, transiently de-differentiate and initiate a genetic program resembling the one found in embryonic pancreatic precursors (recently reviewed in [1,2]). Although numerous studies have identified crucial factors involved in the regeneration of acinar cells, the precise mechanisms initiating the cascade of events leading to their de-differentiation have not yet been identified. Serotonin (5-hydroxytryptamine, 5-HT), a monoamine stored and released by circulating platelets, has long been recognized as a potent bioactive molecule with mitogenic effects not only during embryonic development [3,4] but also in adult cells [5]. Examples of the mitogenic role of 5-HT in adult tissues include liver regeneration following hepatectomy [6], megakaryocyte colony formation [7], pulmonary artery smooth muscle cell proliferation [8] and beta cell expansion during pregnancy [9].

We recently showed that 5-HT plays a major role in the pathophysiology of the exocrine pancreas where it promotes acinar cell secretion and exacerbates the severity of inflammation at the onset of acute pancreatitis [10]. Here we investigated whether 5-HT acts as a mitogen for pancreatic acinar cells and thus promotes tissue regeneration. To this aim, we compared three types of regenerative stimuli, namely cerulein-induced pancreatitis, partial pancreatectomy and thyroid hormone supplementation, in mice deficient in peripheral 5-HT (tryptophan hydroxylase 1 knocked-out, *Tph1*$^{-/-}$), and in wild type mice supplemented with 5-HT precursor.

Material and methods

Animal experiments

All animal experiments were performed in accordance with Swiss federal animal regulations and approved by the cantonal veterinary office of Zurich. Pancreatitis was induced in adult (8-10 weeks of age) wild-type (WT) C57BL/6 (Harlan Laboratories, Horst, The Nederlands) and *Tph1*$^{-/-}$ mice on a C57BL/6 background [11], bred in our facility, via six intra peritoneal (i.p.) injections of 50 μg/kg cerulein administered hourly over a two week period, as described in Supplementary Materials and Methods and [12]. Pancreatectomy was performed according to [13], with the procedure adapted to remove 60% of the organ. 400 μg/kg 3,5,3-L-tri-iodothyronine (T3) was administered daily via i.p. injections for six days. 20 mg/kg 5-hydroxytryptophan (5-HTP) was administered by two subcutaneous injections per day, 12 hours apart. The treatment was started one day before cerulein treatment; control animals received 0.9% NaCl injections.

Histology, immunohistochemistry and immunoblotting

Detailed protocols and primary antibodies used in this study are listed in Supplementary Materials and Methods. Quantification of labelled cells was performed in at least 10 randomly selected high-power fields (×100) per slide. Non-acinar tissue

areas (islets, vessels, fibrotic tissue) were excluded from the analysis.

Electron microscopy analysis

WT and *Tph1*$^{-/-}$ mice were harvested 8 hours after the beginning of cerulein treatment. Sliced pancreata were fixed in 2% glutaraldehyde/1% paraformaldehyde in 0.1M Na/K-phosphate pH 7.4 for 90 min. After washing, the samples were postfixed with 1% osmium tetroxide in 0.1M Na/K-phosphate for one hour, dehydrated in a graded ethanol series, transferred to acetone for embedding in Epon and polymerized at 60°C for 2.5 days. Ultrathin sections were stained with uranyl acetate and lead citrate and examined at an acceleration voltage of 100 kV in a Philips CM 12 transmission electron microscope (Eindhoven, The Netherlands) equipped with a CCD camera (Ultrascan 1000, Gatan, Pleasanton, CA).

Transcript analysis

Total RNA was extracted from pancreata as previously described [14] and reverse transcribed with qScript™ cDNA SuperMix (Quanta Biosciences). Transcript levels were normalized using 18S rRNA as a reference and expressed as fold regulation relative to the value of untreated control animals. Taqman probes (Applied Biosystems) used in this study are listed in Supplementary Materials and Methods.

Results

Lack of 5-HT results in defective acinar cell proliferation and delayed ADM formation

To test whether 5-HT is mitogenic for acinar cells, we compared acinar proliferation following induction of pancreatitis in WT and in *Tph1*$^{-/-}$ mice, characterized by reduced 5-HT levels in the periphery but normal 5-HT signaling in the nervous system [10]. Unexpectedly, *Tph1*$^{-/-}$ animals showed a significantly higher number of acinar cells positive for the general cell cycle activation marker Ki67 after one week of cerulein treatment compared with WT animals, while the number of bromodeoxyuridine (BrdU) positive cells in G1/S phase was similar in the two strains. Phospho-histone H3 (pH3) positive acinar cells in G2/M phase increased in *Tph1*$^{-/-}$ mice only 14 days after the beginning of the treatment (Fig. 1A, S1A). Acinar and interstitial cells were distinguished based on nuclear size, shape and location, as shown in Fig. S1B, C. Quantification of acinar cells in G2 phase, displaying punctuate pH3 staining, and in mitotic phase, with homogeneous nuclear pH3 staining [15], showed a reduced transition into mitotic phase in *Tph1*$^{-/-}$ animals one week after induction of pancreatitis (Fig. 1B).

In addition, formation of acinar-to-ductal metaplasia (ADM), a transient trans-differentiation observed during pancreatitis-induced regeneration, was delayed in *Tph1*$^{-/-}$ mice three days after induction of pancreatitis (Fig. 1C). The slower kinetics of ADM formation was not the result of defective expression or re-localization of β-catenin, a key a transcriptional activator promoting ADM formation [16]. β-catenin was similarly localized on the plasma membrane and nuclei of intact acinar cells in both strains (Fig. 1D) and was also found in ADM of WT animals and in the rare areas with reduced amylase content in *Tph1*$^{-/-}$ mice (Fig. 1E, arrowheads). Seven days after induction of pancreatitis, mature clustered ductal structures were visible in *Tph1*$^{-/-}$ mice and were similar to WT ADMs in terms of β-catenin expression, Sox9 up-regulation, amylase down-regulation and activation of αSma-positive stellate cells (Fig. 2A, S2). Taken together, these data indicate that, upon cerulein stimulation, *Tph1*$^{-/-}$ acinar cells are able to initiate the cell cycle; however, they display defective cell cycle progression and delayed ADM formation.

Lack of 5-HT delays acinar de-differentiation during pancreatitis

Proliferation of pancreatic acinar cells is preceded by a temporary de-differentiation and up-regulation of markers normally associated with pancreatic ductal or progenitor cells [17]. Concomitant with reduced ADM formation, *Tph1*$^{-/-}$ mice showed delayed up-regulation of the ductal marker cytokeratin 19 (Ck19) three days after induction of pancreatitis (Fig. 2A, B). In addition, delayed up-regulation was also observed for several progenitor markers, including *Sox9, Hes1, Notch1, Hnf1β.* Ductal and progenitor gene expression was higher in *Tph1*$^{-/-}$ than in WT animals after 14 days of pancreatitis, with the exception of *Aldh1*, the expression of which was comparable in the two strains (Fig. 2A, B).

Noteworthy, delayed progenitor gene up-regulation in *Tph1*$^{-/-}$ mice was not accompanied by defective down-regulation of genes typical of the differentiated state, such as secretory granule enzymes and transcription factors controlling acinar cell identity, such as Mist-1[18-20] (Fig. S3). These data indicate that WT and *Tph1*$^{-/-}$ mice responded equally to the cerulein stimulus by down-regulating genes specific to mature differentiated acinar cells. However, amylase content did not decrease in *Tph1*$^{-/-}$ acini, as quantified by western blotting (Fig. 3A), enzymatic activity (Fig. 3B) and immunofluorescence analysis (Fig. 3C), indicating that down-regulation of zymogen transcripts was not sufficient to induce zymogen loss in these mice. Autophagy can reduce zymogen content by intracellular degradation [21]. However, autophagy was not differentially regulated in *Tph1*$^{-/-}$ mice, as shown by

comparable processing of the autophagic protein LC3 (Fig. S4A). Similarly, autophagic marker p62 [22] showed widespread expression in acini at 8 hours and down-regulation one day after induction of pancreatitis in both strains (Fig. S4B, C).

Increased zymogen secretion promotes acinar de-differentiation during acute pancreatitis

An alternative cellular mechanism explaining zymogen loss observed in acinar cells following pancreatitis could be the secretion of zymogens into extracellular space. We previously showed that *Tph1*$^{-/-}$ mice have impaired zymogen secretion [10]. This defective secretory process was further investigated at the ultrastructural level. Electron microscopy analysis revealed that zymogen granules were more tightly packed in *Tph1*$^{-/-}$ than in WT mice, both in control and after cerulein treatment (Fig. 3D). Furthermore, size distribution analysis showed that granule size, comparable in the two strains in the untreated controls, increased after stimulation of secretion, indicative of granule swelling preceding content expulsion [23]; however, the enlargement was higher in *Tph1*$^{-/-}$ (Fig. 3E), presumably a consequence of impaired secretion. Thus, our results show that reduced zymogen secretion observed in the absence of 5-HT is associated with defective zymogen loss followed by delayed up-regulation of progenitor genes. To test whether 5-HT-dependent zymogen secretion is required to initiate acinar de-differentiation following pancreatitis, we increased zymogen secretion 24 hours before cerulein administration by treatment with 5-hydroxytryptophan (5-HTP) (regimen scheme depicted in Fig. 4A), a precursor of serotonin that dose-dependently stimulates apical secretion of zymogens into pancreatic ducts (reviewed in [24]). This stimulated secretion is associated with a protective effect during induction of acute pancreatitis [25-27]. Under 5-HTP supplementation and one day of pancreatitis, we also observed lower levels of inflammatory infiltrates in the pancreas (Fig. 4B), chemokine expression (Fig. 4C) and serum amylase (Fig. 4D), likely due to the increased 5-HTP-stimulated apical secretion counteracting the aberrant baso-lateral secretion induced by cerulein. The reduced severity of the disease did not translate in reduced acinar cell de-differentiation, as 5-HTP+cerulein-treated mice showed robust amylase loss in the pancreas similar to cerulein-treated mice (Fig. 4E). In addition, these mice had increased RNA levels of progenitor gene *Hes-1* (Fig. 4F) and earlier expression of Sox-9 in acinar cell nuclei (Fig. 4G). These results suggest that stimulation of zymogen secretion enhances acinar de-differentiation following induction of pancreatitis. However, despite reducing the initial inflammation, one set of 5-HTP supplementation (regimen scheme depicted in Fig. S5A) was not sufficient to promote pancreatic regeneration, as shown by comparable levels of acinar and interstitial cell replication (Fig. S5B) and inflammatory cell infiltration (Fig. S5C) three days after induction of pancreatitis. In addition, increasing the dose of 5-HTP supplementation stimulated the expression of progenitor (Fig. S5D) and inflammatory genes (Fig. S5E) compared to one set of 5-HTP. 5-HTP supplementation over 14 days further boosted the up-regulation of inflammatory genes (Fig. S5F), and resulted in an increased trend of acinar cell replication (Fig. S5G), ADM formation (Fig. S5H) and inflammatory infiltration (Fig. S5I), suggesting that supplementation with exogenous 5-HTP did not ameliorate disease progression.

Lack of 5-HT delays the expression of MMPs during pancreatitis

As inflammatory cells play an active role in ADM formation, we then tested the level of pancreatic inflammation in the two strains. *Tph1*$^{-/-}$ mice showed reduced inflammation after one [10] and three days of pancreatitis (Fig. 4H, S6A). At this time point, expression of type-2 macrophage markers *Mgl1* and *Mrc1* was also reduced in *Tph1*$^{-/-}$ mice, while the type-1 marker *Nos2* was comparable in the two strains (Fig. 4I), Macrophage marker expression increased in *Tph1*$^{-/-}$ mice at later time points (Fig. S6B). Cytokines secreted by macrophages stimulate matrix metalloproteinase (MMP) synthesis and secretion in acinar cells, thus driving digestion of extracellular matrix and ADM formation [28]. Concomitant with reduced macrophage infiltration, *Tph1*$^{-/-}$ mice showed a reduced trend of *Mmp7* (Fig. 4J) and *Tnf* expression (Fig. S6C).

Then we digested the pancreatic extracellular matrix with collagenase and compared de-differentiation of isolated acini after culture in suspension for 24 hours, as described [29]. In this *in vitro* setting, de-differentiation was similar in WT and *Tph1*$^{-/-}$ acini, with or without exogenous 5-HT added to the medium (Fig. 4K), suggesting that the 5-HT mediated effect is exerted up-stream of extracellular matrix digestion.

Analysis of vascular associated factors, including *CD146* and *VEGF*, did not show alterations in the two strains after three days of pancreatitis, when the major ADM difference was observed, but their expression remained elevated in *Tph1*$^{-/-}$ mice after 14 days of the disease (Fig. S6D).

Lack of 5-HT does not alter clonal regeneration of acinar cells following partial pancreatectomy and T3 administration.

As we found that 5-HT supports progenitor-based regeneration following pancreatitis, we then investigated whether zymogen secretion is also required for pancreatic regeneration that does not depend on robust acinar de-differentiation and activation of progenitor genes. To this aim, we analyzed acinar replication following 60% partial pancreatectomy (PPX) that triggers a regenerative response preferentially based on clonal division of differentiated acinar cells. Acinar replication, albeit lower than the one induced after pancreatitis at all the time points analyzed, peaked one week following PPX (Fig. 5A). Importantly, *Tph1*$^{-/-}$ mice did not show accumulation of Ki67 positive acinar cells (Fig. 5A, B). The replication peak was not preceded by robust expression of progenitor genes, the up-regulation of which was lower than during pancreatitis and comparable in the two strains, with the exception of Hes1 that increased at the two week time point only in WT mice (Fig. 5C). In addition, pancreata isolated after pancreatic resection showed comparable amylase content (Fig. 5D) and minimal levels of inflammation, apoptosis and fibrosis in the two strains (Fig.S7A-C). To test the effect of 5-HT in another model of inflammation-independent regeneration, we induced acinar proliferation by administering the thyroid hormone 3,5,3-L-tri-iodothyronine (T3) [30,31]. T3 treated WT and *Tph1*$^{-/-}$ mice showed similar levels of acinar replication (Fig. 5E) and minimal leukocyte infiltration (Fig. 5F). Collectively, these data revealed that acinar replication following PPX and T3 administration is not affected by the lack of 5-HT.

5-HT receptors are up-regulated during pancreatic regeneration

As 5-HT signals via specific 5-HT receptors and transporter, we then compared their expression during pancreatic regeneration. Type 1 and 2 receptors, known to play a role in regeneration of liver and heart [6,32,33] and found up-regulated in pancreatic cancer [34], were up-regulated following pancreatitis in WT mice and showed a delayed kinetic in *Tph1*$^{-/-}$ animals (Fig. S8A). Analysis of samples with reduced inflammation, such as *Tph1*$^{-/-}$ and 5-HTP supplemented WT mice one day after pancreatitis, indicated that inflammatory cells substantially contribute to the observed level of receptor expression, except in the case of *5-HT2A* (Fig. S8B). Similarly, PPX samples, characterized by minimal tissue inflammation, showed lower 5-HT receptor and transporter expression compared with pancreatitis specimens (Fig. S8C). Interestingly, expression of *5-HT2B* robustly increased in WT mice 14 days after PPX.

To test whether acinar cells also express 5-HT receptors and transporter, we isolated pancreatic acini one day after pancreatitis induction. In WT acinar cells, expression of 5-HT receptors, but not transporter, increased upon disease induction. *Tph1*$^{-/-}$ acini had an opposite trend of receptor expression compared with WT cells, showing lower type 1 and higher type 2 receptor levels (Fig. S8D). Collectively, these results indicate that pancreatic regeneration is associated with up-regulation of 5-HT receptors and transporter, which can be expressed not only by infiltrating cells but also by pancreatic acini.

Hes1-Ptf1a axis is activated in the uninjured adult pancreas

As we observed that 5-HTP treatment enhanced acinar de-differentiation following induction of pancreatitis (Fig. 4E, F), we then tested whether this regulation was observed also in the absence of tissue damage. 5-HTP-treated WT mice had normal levels of serum and tissue amylase (Fig. 4C, D) and no Sox9 expression in acinar cells (Fig. 6A), suggesting that increased secretion into ducts is not sufficient to activate acinar de-differentiation. However, 5-HTP treatment increased the expression of the progenitor gene *Hes1* in the pancreas (Fig. 6B), albeit at a lower level than following 5-HTP+cerulein treatment. Hes1 is a critical orchestrator of pancreatic embryogenesis (reviewed in [35]), as it regulates maintenance of progenitor cells and timing of cell differentiation by antagonizing the function of Ptf1a, a basic helix-loop-helix transcription factor that promotes zymogen synthesis [36]. *Hes1* up-regulation following 5-HTP treatment was concomitant with *Ptf1a* down-regulation (Fig. 6B). Moreover, early time points following cerulein treatment showed initial *Ptf1a* up-regulation followed by *Hes1* expression and subsequent *Pft1a* down-regulation. 24 hours after induction of pancreatitis, *Ptf1a* levels were higher in *Tph1*$^{-/-}$ mice with delayed *Hes1* up-regulation (Fig. 6C). This suggests that the inhibitory function of Hes1 on Ptf1a expression described during development may be active also in the adult pancreas. We further explored the Hes1-Ptf1a regulation in an *in vitro* system where the acinar cell line AR42J was stimulated toward a more differentiated and secretory phenotype by treatment with dexamethasone [37]. 48 hours of dexamethasone treatment changed the cell morphology from a more spheroid to flattened shape (Fig. S9A), led to decreased replication of AR42J cells (Fig. S9B), increased amylase content (Fig. 6D) and decreased *Sox-9* expression (Fig. 6E). Similar to what we observed *in vivo*, AR42J differentiation showed an initial increase of *Ptf1a* expression that was then reduced following *Hes1* up-regulation (Fig. 6F).

Taken together, the *in vivo* and *in vitro* results presented here show that conditions of increased zymogen secretion and synthesis in acinar cells

are associated with the activation of the Hes1-Ptf1a axis. These data not only indicate that different progenitor genes are regulated in a different manner in acinar cells, but also that the Hes1-Ptf1a axis may contribute to maintain physiological zymogen levels in the adult pancreas in the absence of acinar cell damage.

Discussion

The remarkable regenerative capacity observed in organs of several non-mammalian vertebrates often involves the de-differentiation of mature cells (reviewed in [38]). Notably, cell de-differentiation is also observed in the regeneration of the adult exocrine pancreas of mammals following pancreatitis. In this organ, differentiated acinar cells transiently revert into a progenitor state following injury, thus likely acting as facultative progenitor cells and initiating a replicative program leading to exocrine tissue repair (reviewed in [1,39,40]). This de-differentiation of adult pancreatic acinar cells is a critical process within the regenerative mechanism, as *bona fide* resident stem cells have not been reported in the adult pancreas (reviewed in [39]). Our results showed that, differently from what has been observed in other cell types and tissues [6,33,41-46], 5-HT does not act as a strong mitogen for acinar cells, as acinar replication was unchanged *in vivo* in the absence of 5-HT following 60% pancreatectomy and T3 stimulation and *in vitro* in AR42J acinar cells treated with 5-HT (data not shown). However, 5-HT is required for acinar de-differentiation following inflammatory-mediated tissue damage. In this context, it is important to mention that 60% pancreatectomy is characterized by a moderate level of acinar cell de-differentiation and replication compared with the more robust regeneration observed during induction of pancreatitis. These observations support the notion that, according to the type and extent of tissue injury, the mammalian pancreas has the ability to trigger different regenerative mechanisms, which are driven by activation of a progenitor-like program or by a simple, clonal proliferation of differentiated acinar cells (reviewed in [39,47]). Furthermore, as progenitor-based and clonal modalities of regeneration are not mutually exclusive and are likely to co-exist, the clonal regeneration independent from zymogen secretion is likely to account for the replication observed in *Tph1*$^{-/-}$ mice with compromised progenitor-based regeneration.

A major challenge undertaken in our study was to elucidate the mechanisms by which 5-HT affects acinar de-differentiation and ADM formation. We identified 5-HT-dependent secretion of zymogen content as one of the step promoting up-regulation of progenitor-like genes, suggesting that physical removal of zymogens via secretion has to occur to allow de-differentiation of adult acinar cells. However, additional mechanisms are likely to contribute to the observed delayed de-differentiation and ADM formation in the absence of 5-HT. We found that reduced secretion in *Tph1*$^{-/-}$ mice limits chemokine production and leukocyte infiltration in the early stages of cerulein-induced pancreatitis ([10] and this work). Thus, it is possible that the few infiltrating leukocytes released a limited amount of pro-inflammatory cytokines that may not have been sufficient to stimulate acinar de-differentiation and ADM formation. Indeed, *Tph1*$^{-/-}$ mice had reduced levels of *Tnf*α and *MMP*, both factors critical to induce ADM formation [28]. The comparable de-differentiation observed *in vivo* in isolated acini supports the hypothesis that the 5-HT effect is not acinar cell autonomous but is mediated by macrophage-stimulated extracellular matrix digestion. However, it has to be noted that, despite sharing phenotypic similarities, the *in vitro* acinar de-differentiation may not completely reflect the molecular mechanisms occurring in the *in vivo* de-differentiation upon pancreatitis induction. Indeed, the process of acinar isolation *per se* activates Ras and its down-stream effectors ERK and AKT [29], the activity of which promotes cell de-differentiation. Thus, it is possible that the *in vitro* procedure bypasses a cell autonomous, 5-HT-mediated process occurring *in vivo*. In support of this hypothesis, 5-HT has been reported to act upstream Ras-dependent ERK1/2 activation via binding to different 5-HT receptors [48-50].

In this regard, an alternative explanation for the aberrant progenitor-based regeneration observed in *Tph1*$^{-/-}$ mice is that 5-HT directly stimulates the expression of progenitor genes via 5-HT receptor signaling or intracellular mechanisms following its uptake. We found that type 1 and 2 5-HT receptors were up-regulated during pancreatic regeneration, following both pancreatitis and PPX. Of note, type-1 5-HT receptors were recently described to promote proliferation of human pancreatic cancer cells [34]. Finally, we also discovered an activation of the Hes1-Ptf1a axis in the uninjured pancreas. Both under *in vivo* and *in vitro* experimental conditions, *Hes1* up-regulation was accompanied by down-regulation of *Ptf1a* expression. This suggests the presence of a tightly regulated control of Ptf1 expression by Hes1 in the adult pancreas, reminiscent of the embryonic situation. Hence, our data are in support of a dual role of Hes1 in the pathophysiology of the pancreas. In physiological situations, increased zymogen secretion is associated with moderate Hes1 up-regulation, which limits Ptf1a expression and thus contributes to acinar homeostasis by preventing excessive zymogen expression. In pathological

situations that trigger acinar replication, a higher Hes1 up-regulation occurs with a consequent reduction of transcription factors and proteins required to maintain acinar function. This down-regulation of differentiation genes may provide the cells with the suitable environment to up-regulate a broader set of progenitor genes, leading to a robust acinar proliferation and organ repair. Collectively, these results indicate that expression of progenitor genes is not a synchronous event. Therefore, similarly to what was observed in the determination of diverse cell fates during development [51], the different time of expression of these transcription factors in the adult pancreas may reflect their distinctive function during homeostasis and regeneration.

While highlighting the impact that 5-HT exerts on the de-differentiation and proliferation abilities of pancreatic cells under different regenerative conditions, this study nevertheless has some limitations. First, the use of a general *Tph1* knocked out model system precludes the dissection of the individual cell contribution in the synthesis of 5-HT. Serotonin is mainly produced by intestinal enterochromaffin cells and stored and released by circulating platelets. However, additional sources of serotonin exist, including nerve fibers, which are abundant in the pancreatic tissue, β-cells [52] and even acinar cells [53]. In addition, further studies are necessary to characterize the role of the different 5-HT receptors present on acinar and non-acinar cells to fully understand their contribution during pancreatic regeneration. Finally, an aspect of the 5-HT biology that has not been addressed in this study is a thorough characterization of vascular function. Disturbances in microcirculation, including vasoconstriction, are associated with acute pancreatitis (reviewed in [54]). As 5-HT is a potent vasoconstrictor, it will be worth exploring whether alterations in the vascular biology contribute to the observed phenotype in *Tph1*$^{-/-}$ mice.

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Contributors The authors of this manuscript contributed in the study design, acquisition, analysis, interpretation of data, drafting and critical revision of the manuscript. ES, KG, MB, RB, ABS, EM, GS performed experiments, generated and analyzed data; ABH performed confocal microscopy, generated and analyzed data; EMS performed electron microscopy; YT performed partial pancreatectomy; AZ performed experiments and generated data; TR generated transgenic lines; RG, SS designed the study; ES, SS wrote the manuscript; RG, ABS revised the manuscript for content, analysis and interpretation of data. All authors approved the submitted version.

List of abbreviations ADM, acinar-to-ductal metaplasia; *Tph1*$^{-/-}$, tryptophan hydroxylase 1 knocked-out; PPX, 60% partial pancreatectomy; 5-HTP, 5-hydroxytryptophan; T3, 3,5,3-L-tri-iodothyronine.

Data sharing statement: no additional data

List of online supporting material:
Supplementary material and methods and four supplementary figures

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Figures

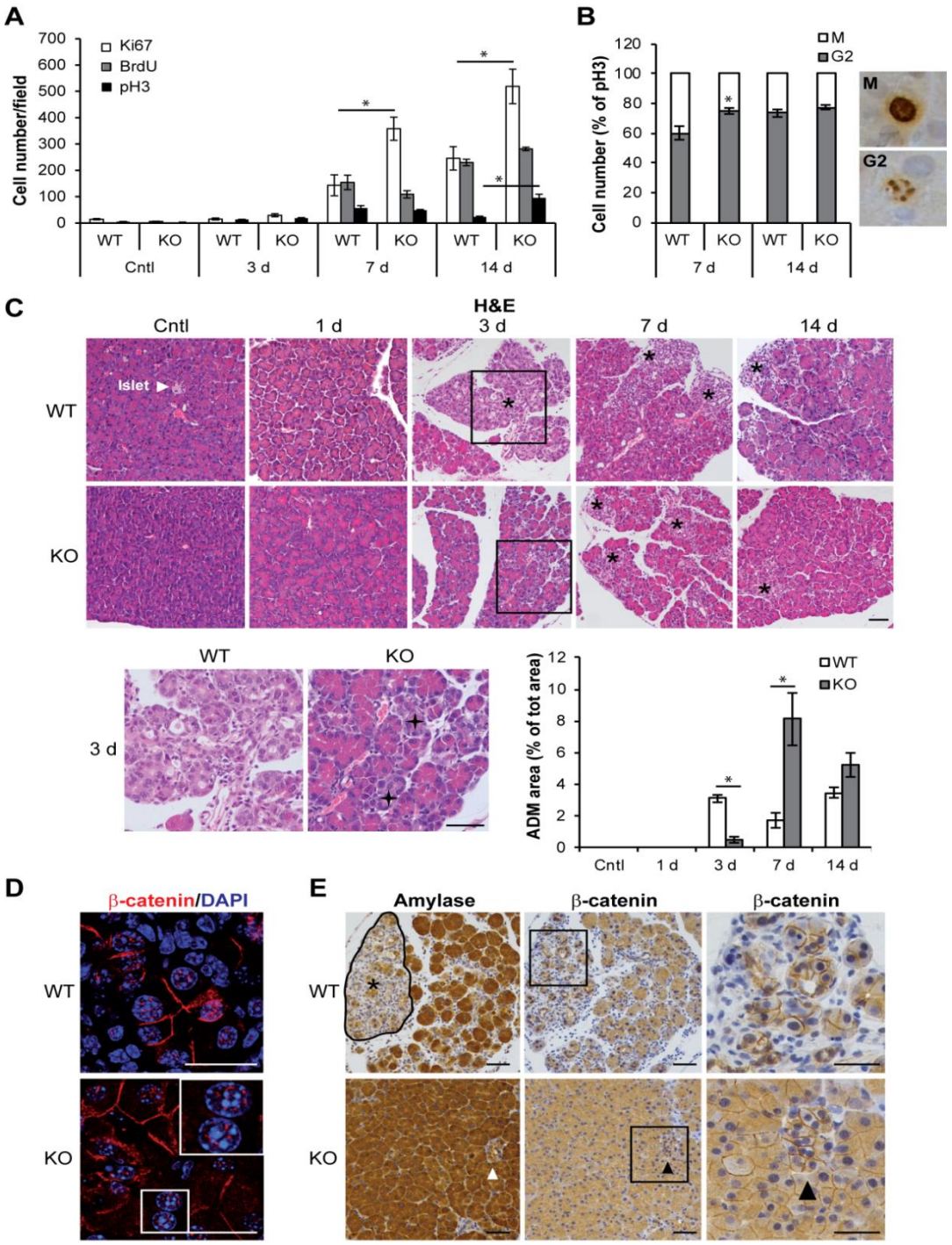

Figure 1. Lack of 5-HT results in defective acinar cell proliferation and delayed ADM formation. **A**. Quantification of replication markers expressed in pancreatic acinar cells showed increased number of Ki67 positive cells in *Tph1*$^{-/-}$ mice after 7 and 14 days of pancreatitis. **B**. Relative amount of acinar cells in G2 and M phases determined by pH3 staining pattern 7 and 14 days after pancreatitis induction. Data are expressed as percentage of the total number of pH3 positive cells with punctate (G2 phase) or complete nuclear staining (M phase). **C**. Hematoxylin-Eosin (H&E)-staining and ADM quantification showed lower amount of ADM (asterisks) in *Tph1*$^{-/-}$ mice after three days of pancreatitis induction. Lower panels: enlarged views of insets three days after pancreatitis. Note the presence of mature ADM in WT mice characterized by loss of acinar content and tubular complex formation. *Tph1*$^{-/-}$ pancreata presented sporadic de-regulation of acinar content (stars) without re-organization into tubular structures. **D**. Single plane confocal images showing nuclear localization of β-catenin in WT and *Tph1*$^{-/-}$ pancreata three days after induction of pancreatitis. Nuclei are stained with DAPI (blue). **E**. Amylase and β-catenin staining three days after induction of pancreatitis. Circled area and asterisk in WT pancreata represent ADM. Right panels: enlarged views of insets. Arrowhead, area with reduced amylase content and increased β-catenin expression in *Tph1*$^{-/-}$ pancreata. Results are average ± SEM (n=5), *p<0.05. Scale bars: 50 µm

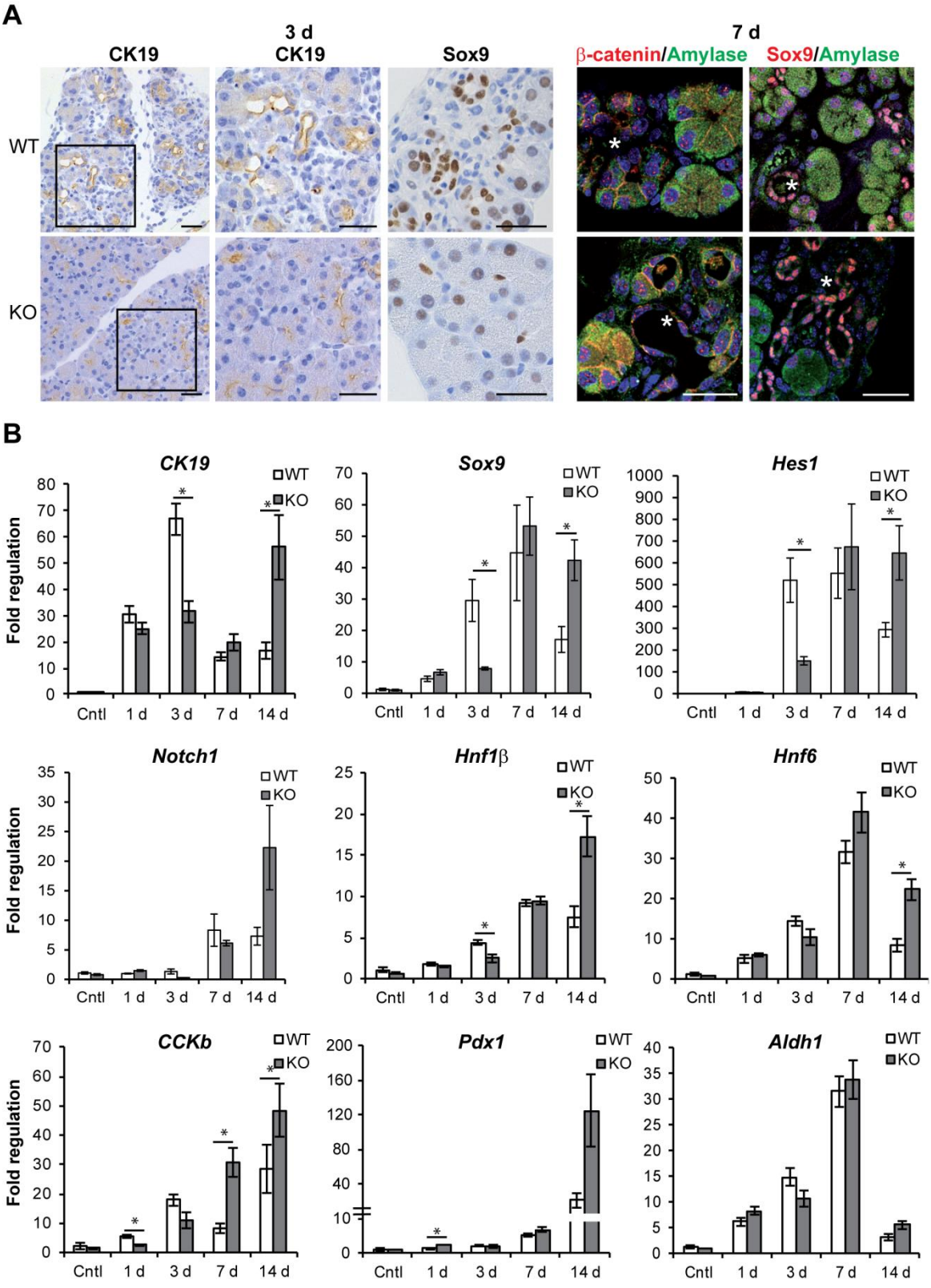

Figure 2. Lack of 5-HT delays acinar de-differentiation during pancreatitis. **A**. Cytokeratin 19 (Ck19), Sox9, β-catenin and amylase staining three and seven days after induction of pancreatitis. Nuclei in the IF pictures are stained with DAPI (blue). **B**. qPCR of ductal and progenitor markers during the indicated times of cerulein-induced pancreatitis showed delayed expression in *Tph1*$^{-/-}$ pancreata. Results are average ± SEM (n=5), *p<0.05. Scale bars: 50 μm.

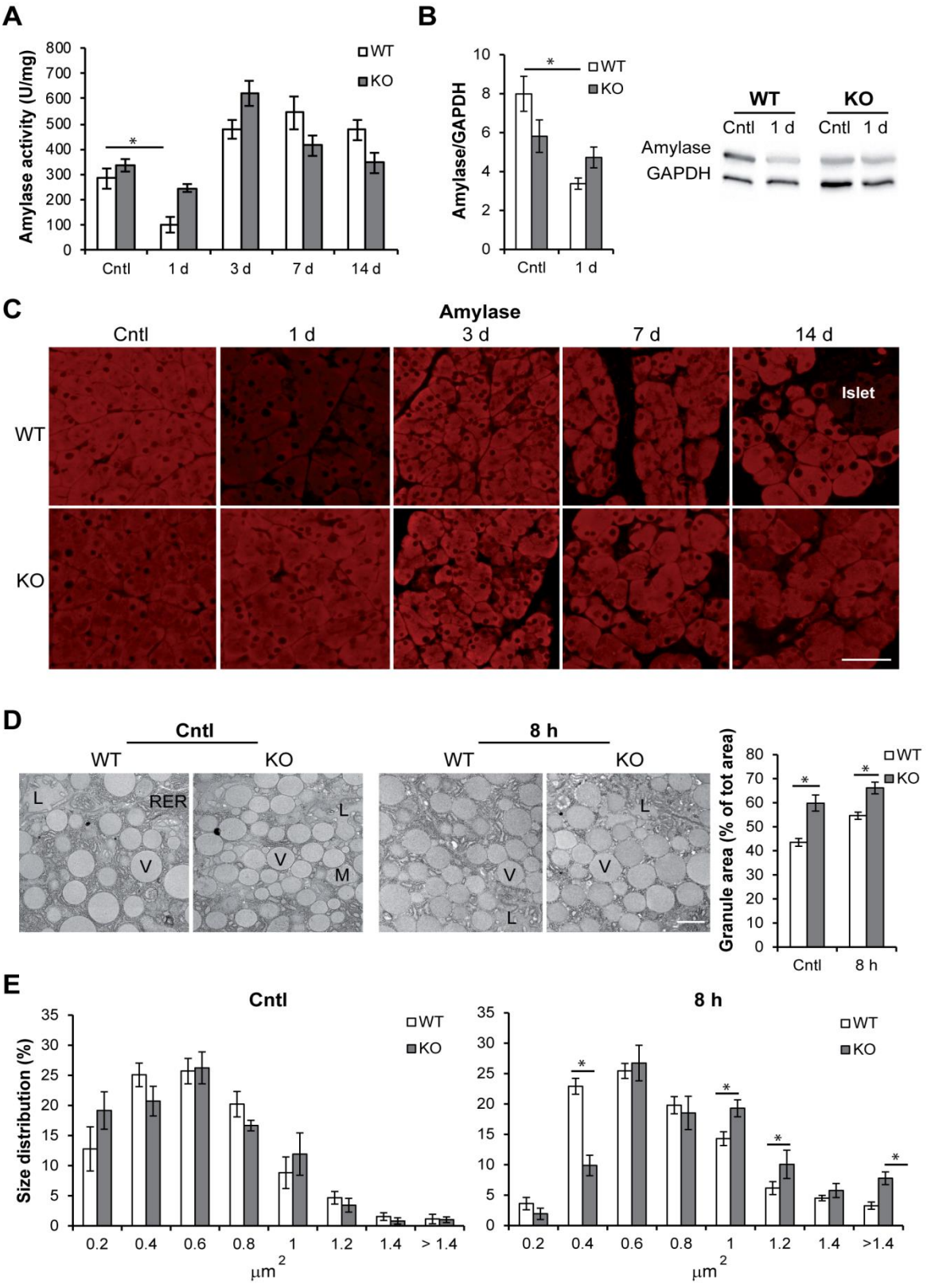

Figure 3. Lack of 5-HT prevents loss of amylase during pancreatitis. Biochemical determination of amylase activity (**A**) and immunoblotting of 10 µg of proteins (**B**) showed down-regulation of amylase in pancreatic tissue of WT but not *Tph1*$^{-/-}$ mice one day after cerulein treatment. **C**. Immunostaining of amylase during 14 days of pancreatitis induction showed decreased protein expression in WT but not in *Tph1*$^{-/-}$ mice one day after cerulein treatment. **D**. Electron micrographs of WT and *Tph1*$^{-/-}$ pancreata. Note the close proximity of zymogen granules with reduced inter-granule space in *Tph1*$^{-/-}$ mice, both in control and cerulein treatment. L, acinar lumen with evident microvilli. V, zymogen granule, M, mitochondrion. RER, rough endoplasmic reticulum. Right panel, quantification of the area occupied by granules expressed as percentage of total area. **E**. Size distribution of zymogen vesicle diameter. Note the higher abundance of vesicles with large diameter in *Tph1*$^{-/-}$ mice following pancreatitis induction. Results are average ± SEM (n=5), *p<0.05. Scale bars: 1 μm.

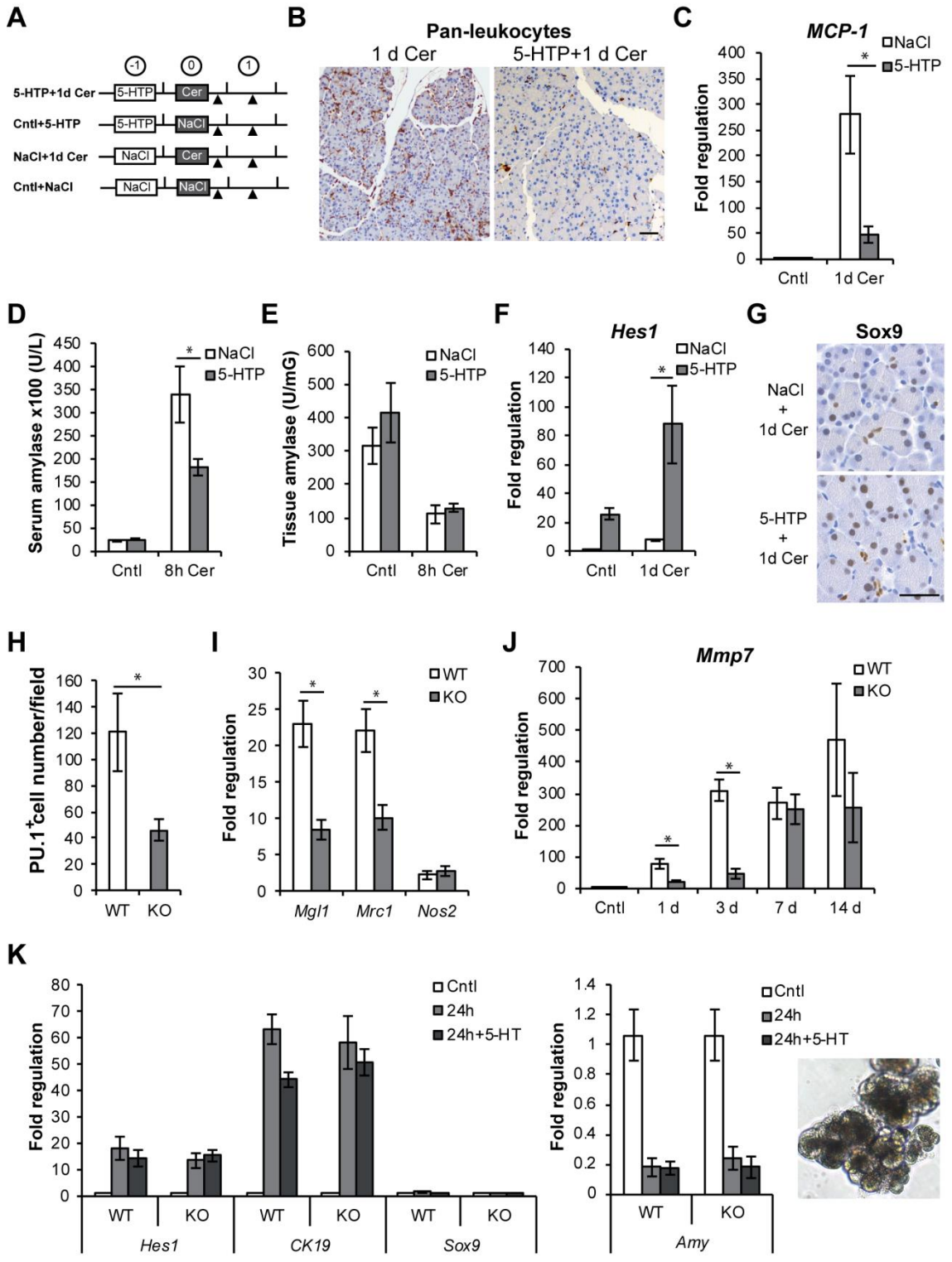

Figure 4. **A**. Scheme depicting the combined regimen with 5-HTP and cerulein (Cer). 0.9% NaCl was used as vehicle control. Circles indicate days of treatment starting from the first cerulein injection and triangles indicate days of animal harvest. Pan-leukocyte immunostaining with anti-coronin 1 (**B**) and qPCR of pro-inflammatory chemokine MCP-1 (**C**) showed lower inflammation upon 5-HTP supplementation one day after induction of pancreatitis. Serum (**D**) and tissue (**E**) levels of amylase 8 hours after pancreatitis induction following 5-HTP supplementation. qPCR of *Hes1* (**F**) and immunostaining of Sox9 (**G**) one day after pancreatitis induction following 5-HTP supplementation. **H**. inflammatory cell quantification after PU.1 immunostaining showed reduced infiltration in *Tph1*$^{-/-}$ mice three days after pancreatitis induction. **I**. qPCR of type1 and 2 macrophage markers three days after pancreatitis induction. **J**. qPCR of matrix metalloproteinase 7 (*Mmp7*) at the indicated days of pancreatitis induction. **K**. qPCR of progenitor and zymogen markers in isolated acini (inset) cultured for 24 hours in suspension to induce differentiation. 5-HT was added to the medium at 20 μM concentration. Results are average ± SEM (n=5), *p<0.05. Scale bars: 50 μm.

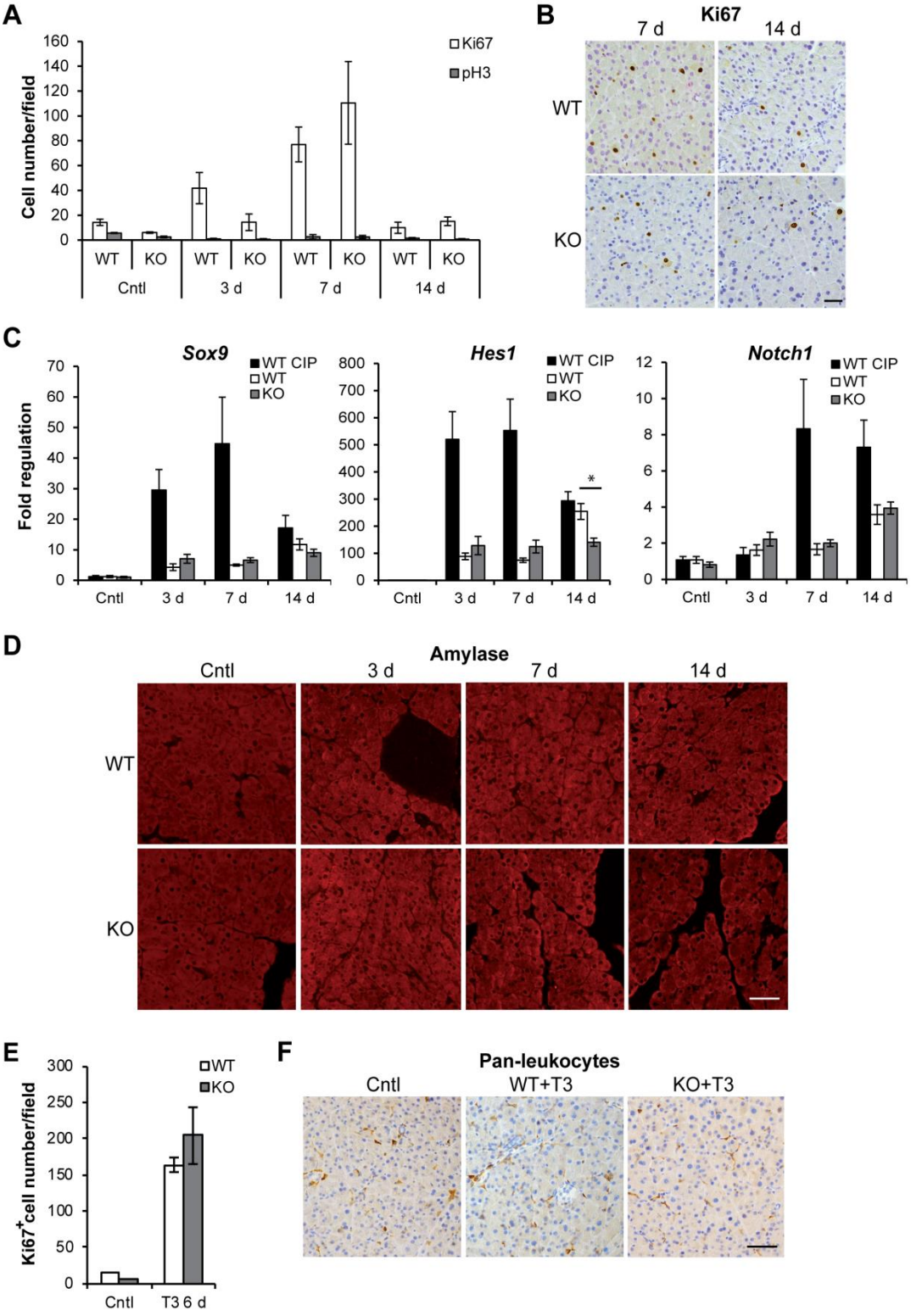

Figure 5. Lack of 5-HT does not alter clonal regeneration of acinar cells following 60% pancreatectomy (PPX) and thyroid hormone T3 stimulation. **A**. Quantification of replication markers expressed in pancreatic acinar cells at the indicated times after partial pancreatectomy. **B**. Immunostaining of Ki67 at the indicated time points following PPX. Pictures represent an inset (1:10) of the original images used for Ki67 quantification. **C**. qPCR of progenitor-cell markers at the indicated times after PPX. Note that WT but not *Tph1*$^{-/-}$ mice increased *Hes1* expression 14 days after surgery. Expression levels obtained in WT mice after cerulein induced pancreatitis (WT CIP) are shown as a comparison. **D**. Immunostaining of amylase showed comparable protein content in WT and *Tph1*$^{-/-}$ mice following PPX. **E**. Quantification of Ki67 expressed in pancreatic acinar cells following T3 administration for six days. **F**. Pan-leukocyte immunostaining with anti-coronin1 showed comparable level of inflammatory cells in control and treated mice. Results are average ± SEM (n=3-5), *p<0.05. Scale bars: 50 μm.

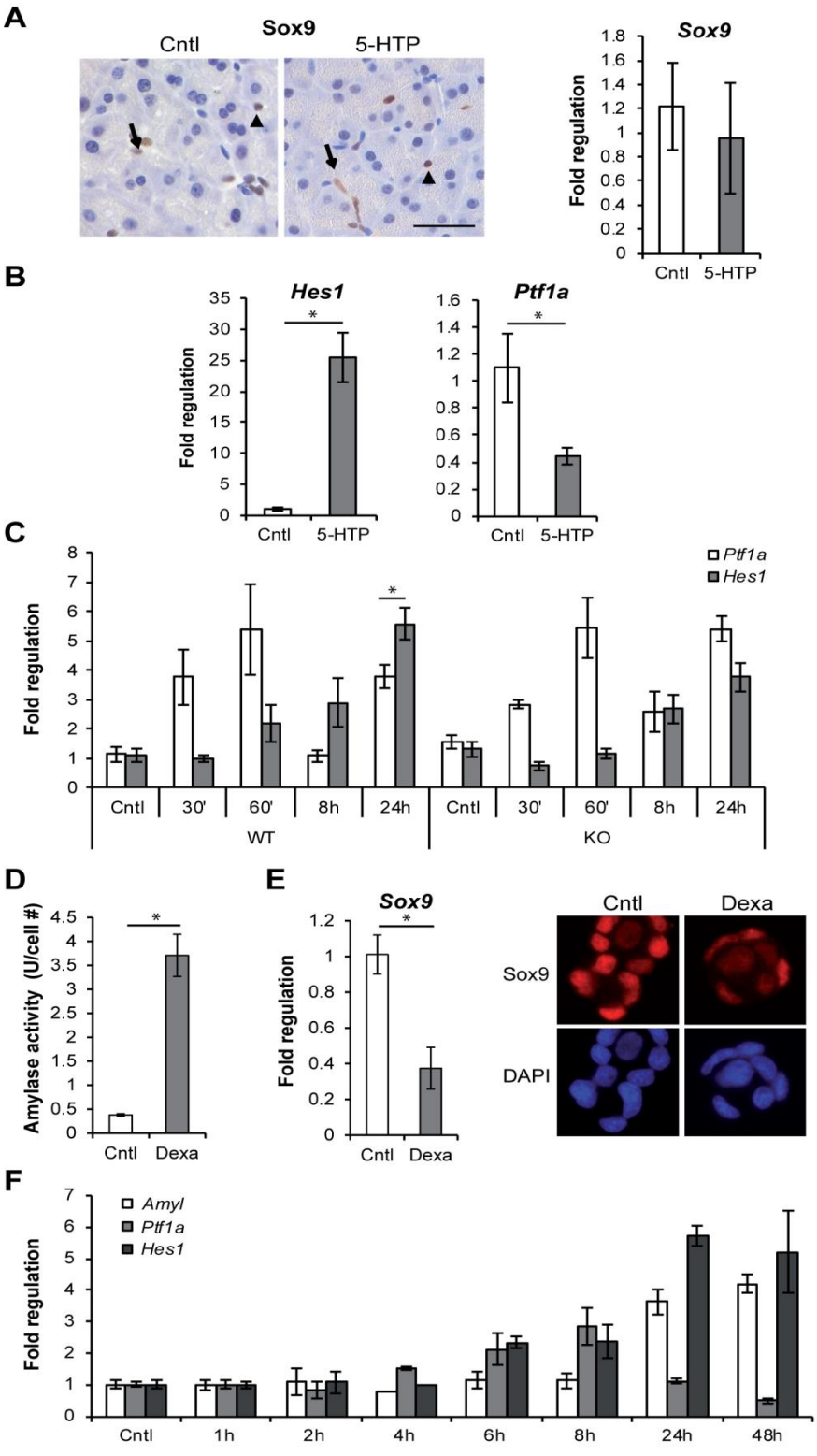

Figure 6. Increased zymogen secretion stimulates *Hes1* expression in the uninjured pancreas. **A**. Left panel, immunostaining of Sox9 following 5-HTP supplementation showed nuclear expression of the protein in interstitial and centro-acinar cells but not in acinar cells. Right panel: qPCR analysis showed comparable levels of *Sox9* transcripts in control and 5-HTP-treated pancreata. **B**. qPCR analysis showed *Hes1* up-regulation and *Ptf1a* down-regulation in mouse pancreata following 5-HTP treatment. **C**. qPCR of *Ptf1a* and *Hes1* expression in WT and *Tph1*$^{-/-}$ mice in the early phases after pancreatitis induction. Dexamethasone treatment of pancreatic acinar AR42J cells for 48 hours increased cellular content of amylase (**D**) and reduced Sox9 expression at both transcript (left panel) and protein (right panel) levels (**E**), indicative of increased cellular differentiation. **F**. qPCR analysis showed a time-dependent increase of *Hes1* a decrease of Ptf1a expression in dexamethasone-treated cells. Results are average ± SEM (n=5), *p<0.05. Scale bars: 50 µm.

Supplementary Online Materials

Materials and Methods

Biochemical reagents

Unless otherwise stated, all chemicals were purchased from Sigma and cell culture reagents from Gibco-BRL. Stock solution for 5-HTP and cerulein were made in NaCl, dexamethasone in F-12K medium.

Pancreatitis induction

Animals received six hourly injections of 50 µg/kg cerulein on Monday, Wednesday and Friday and were harvested on Tuesday after one set of injections (day 1), on Thursday after two sets of injections (day 3) or on Monday after three (day 7) or six (day 14) sets of injections, without receiving cerulein treatment on the same day.

Pancreatic cell culture

Pancreatic acini were isolated from WT and *Tph1*$^{-/-}$ mice was performed as previously described in [55]. Cells de-differentiaion was induced according to [56] by culturing isolated acini in suspension for 24 hours in RPMI 1640 glutamax medium supplemented with 10% fetal bovine serum, 50 U/mL penicillin, 50 µg/mL streptomycin, 0.1 mg/ml soybean trypsin inhibitor. 20µM 5-hydroxytryptamine creatinine sulfate complex (Sigma-Aldrich, Buchs, Switzerland) or PBS as vehicle control was added to the medium.
AR42J cells were maintained in Kaighn's modified Ham's F-12 medium with 20% fetal bovine serum (FBS), 50 U/mL penicillin, and 50 µg/mL streptomycin at 37°C in a 5% CO_2 atmosphere. Differentiation was induced by incubating adherent cells with 50 nM dexamethasone.

Biochemical analysis of amylase activity

For determination of amylase levels present in the serum, blood was sampled by heart puncture. For determination of amylase levels present in pancreatic tissue, pancreata were homogenized with RIPA buffer containing protease inhibitor cocktail (complete ultra-tablets mini, Roche Diagnostics, Mannheim, Germany). Amylase activity was measured using the Fuji Dri-Chem 4000i analyzer (Glattbrugg, Switzerland).

Immunohistochemistry

Pancreas specimens were embedded in paraffin for histological analyses as described [57]. For immunofluorescence analysis of cultured cell lines, AR42J cells were fixed in 3.6% formaldehyde and permeabilized with 0.2% Triton X-100 in PBS. Primary antibodies used in this study were: rabbit anti-phospho-histone 3 (Millipore, Massachusetts, USA), rabbit anti-sox9 (Millipore, Massachusetts, USA), rabbit anti-cyclin E (Abcam, Cambridge, UK), rabbit anti-coronin 1 (gift from Jean Pieters), rabbit anti-amylase (Sigma-Aldrich, Buchs, Switzerland), rabbit anti Ki67 (Abcam, Cambridge, UK), rabbit anti-p62/SQSTM1 (MBL, Massachusetts, USA), rabbit anti-β-catenin (Cell Signaling, Danvers, USA), rabbit anti-PU.1 (Cell Signaling, Danvers, USA), mouse anti-αSMA (Dako, Glostrup, Denmark), goat anti-amylase (Santa Cruz Biotechnology, California). Secondary antibodies used in this study were: Biotinylated Goat Anti-Rabbit IgG (H+L). This antibody is included in the VECTASTAIN® ABC kits, AlexaFluor 594, Goat Anti-Rabbit IgG (Life Technologies, Carlsbad, California, USA), FITC anti-goat (AdB Serotec, Germany), FITC anti-mouse (AdB Serotec, Germany). Nuclei were visualized with 4′, 6-diamidino-2-phenylindole (DAPI). Detection of proliferating cells in s-phase was performed with Invitrogen BrdU Staining Kit (Life Technologies, Carlsbad, California, USA). Detection of DNA fragmentation in apoptotic cells was performed with a TUNEL assay using an ApopTag peroxidase Kit (MP Biomedicals, Illkirch, France). Microscopy analyses were performed on a Nikon Eclipse Ti fluorescence microscope (Amsterdam, The Netherlands) or on a Leica SP2 AOBS confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany), using the appropriate settings. Image stacks of optical sections were further processed using the Huygens deconvolution software package version 2.7 (Scientific Volume Imaging, Hilversum, NL).

Western blotting

Immunoblotting was performed by homogenizing tissue samples in RIPA buffer containing protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Protein concentrations were determined using a Bradford protein assay (BioRad, Hercules, CA, USA). Aliquots corresponding to 10 µg of proteins were separated by SDS-PAGE electrophoresis. Blotting and chemiluminescent detection of immunoreactive bands were performed using the V3 Western Workflow system (BioRad, Hercules,

CA, USA), according to the manufacturer protocols. Primary antibodies rabbit anti-amylase (Sigma-Aldrich, Buchs, Switzerland), rabbit anti-LC3B (Cell Signaling, Danvers, USA), and rabbit anti GAPDH (Sigma-Aldrich, Buchs, Switzerland) were incubated overnight at 4^{o}C.

Transcript analysis

The following Taqman probes (Applied Biosystems) were used: *Sox9* Mm00448840_m1, *Hes1* Mm01342805_m1, *Notch-1* Mm00435249_m1, *Mist1* (Bhlhb8) Mm00487695_m1, *Cpa1* Mm01137017_m1, *MCP1/CCL2* Mm00441242_m1, *PTF1a* Mm00479622_m1, *Amy* Mm00651524_m1, *Aldh1a1* Mm00657317_m1, *Hnf1b* Mm00447459_m1, *Hnf6* (ONECUT1) Mm00839394_m1, *Ck19* Mm00492980_m1, *Cckbr* Mm00432329_m1, *CD146* (*Mcam*) Mm00522397_m1, *Htr1a* Mm00434106_s1, *Htr1b* Mm00439377_s1, *Htr2a* Mm00555764_m1, *Htr2b* Mm00434123_m1, *SERT* (Slc6a4) Mm00439391_m1, *Mao A* Mm00558004_m1, *Mgl1* (*Clec 10a*) Mm00546124_m1, *Nos2* Mm00440502_m1, *Mrc1* (*MMR, Clec 13D*) Mm00485148_m1, *Mmp7* Mm00487724_m1, *Pdx1* Mm00435565_m1, *VEGFα* Mm00437304_m1, *IL-1β* Mm00434228_m1, *IL-6* Mm00446190_m1, *Tnfα* Mm00443258_m1, *Amy2* Rn00821330_g1, *Ptf1a* Rn00588645_m1, *Hes1* Rn00577566_m1, *Sox9* Rn01751069_m1,TaqMan Ribosomal RNA Cont Reagents VIC (18S).

Statistical analyses

Groups of 5-6 animals were tested for each experiment. The data are expressed as the means ± SEM. The statistical significance of differences in the means of experimental groups was determined using an unpaired, two-tailed Student's *t* test unless otherwise stated. A p value < 0.05 was considered to be significant.

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Figures

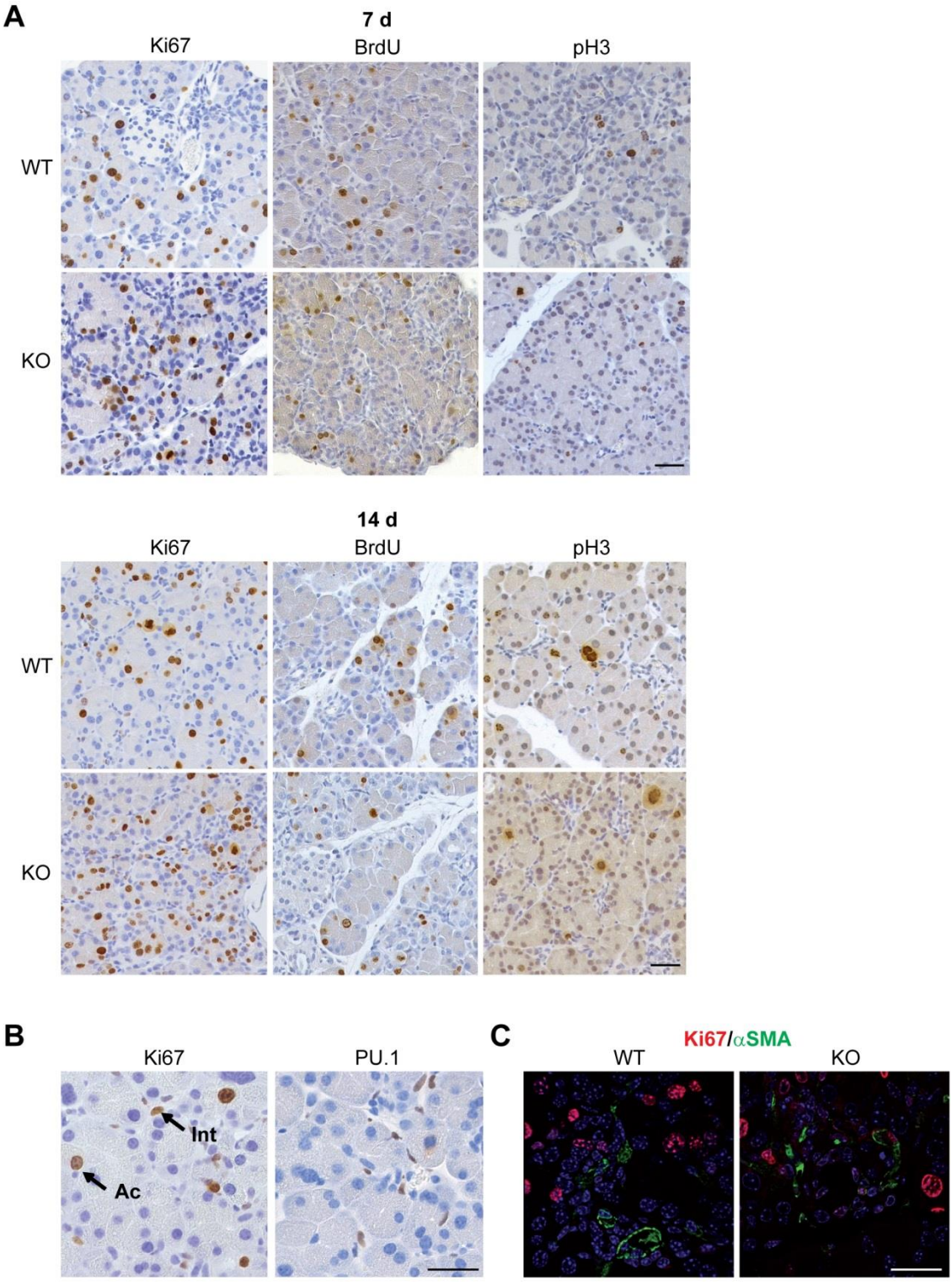

Figure S1. **A**. Immunostaining of Ki67, 5-bromo-2'-deoxyuridine (BrdU) and phospho-histone 3 (pH3) at the indicated time points following cerulein treatment. Pictures represent an inset (1:10) of the original images used for quantification of positive cell number. **B**. Acinar cells (Ac) positive for Ki67 were identified by large and round nuclei located inside the acinar periphery, while interstitial cells (Int) had smaller and elongated nuclei present outside the pancreatic acini. PU.1 staining confirmed that part of interstitial cells with elongated nuclei was of inflammatory origin. Limited Ki67-αsmooth muscle actin (αSma) co-staining showed that interstitial activated stellate cells were not highly replicative both in WT and *Tph1*$^{-/-}$ mice. Scale bars: 50 μm.

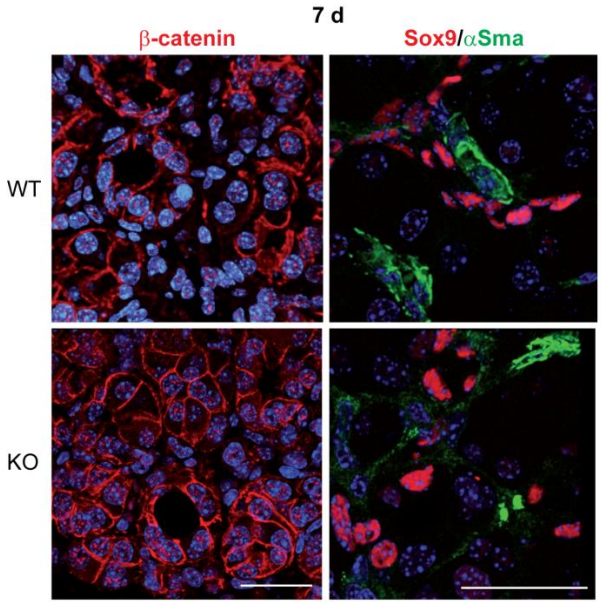

Figure S2. β-catenin, Sox9 and αsmooth muscle actin (αSma) immunostaining 7 days after induction of pancreatitis. Nuclei are stained with DAPI (blue). Scale bars: 50 μm.

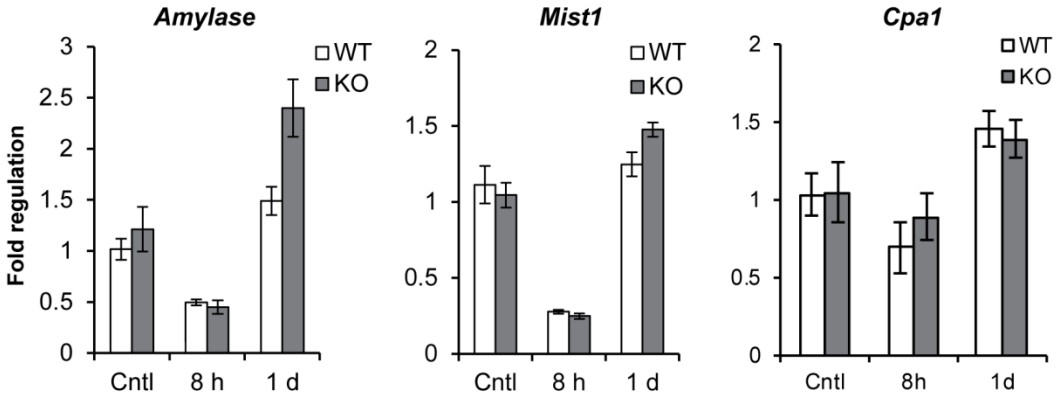

Figure S3. qPCR of genes typical of the differentiated state showed similar expression in the two strains 8 hours after cerulein treatment. *Cpa1*, carboxypeptidase 1. Results are average ± SEM (n=5).

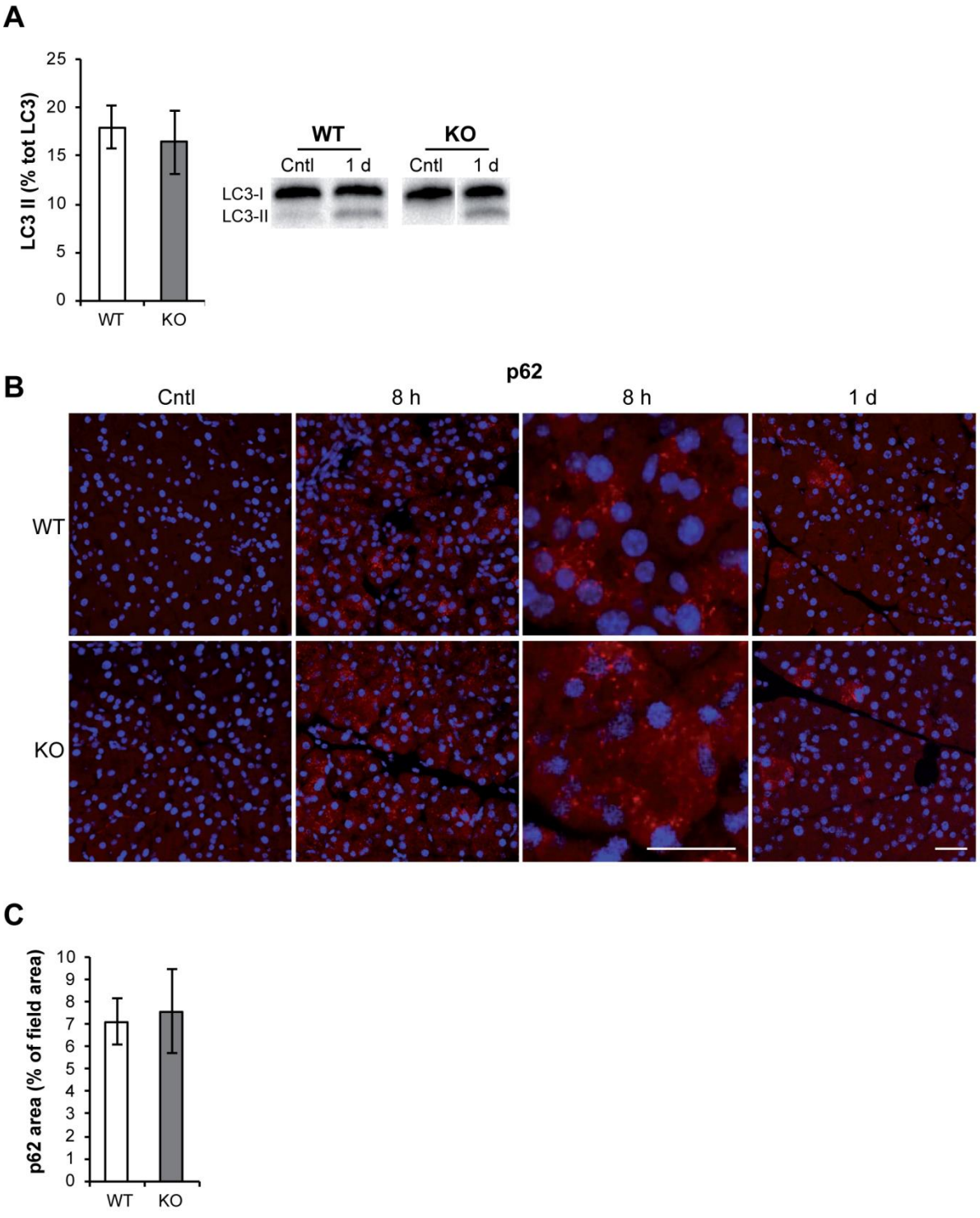

Figure S4. **A**. Immunoblotting and quantification of processed LC3 (LC3-II) one day after cerulein treatment. Data are expressed as percentage of total LC3 (I+II). Results are average ± SEM (n=4). **B**. Immunostaining of the autophagic marker p62 at the indicated time points following cerulein treatment. **C**. Quantification of p62 positive areas one day after cerulein treatment. Data are expressed as percentage of the pancreatic area in the field. Scale bars: 50 μm.

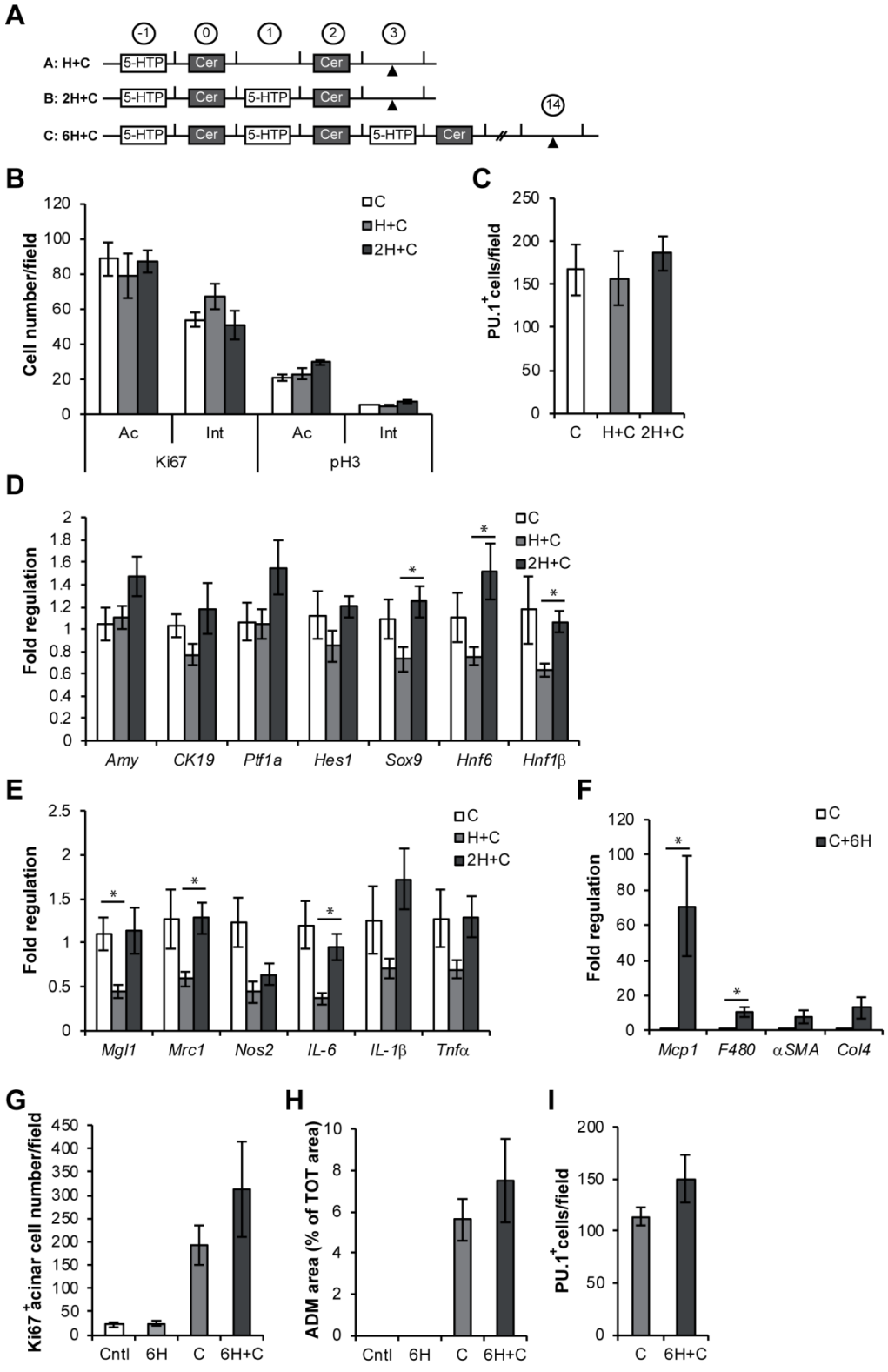

Figure S5. **A**. Scheme depicting the treatment regimen with 5-HTP and cerulein (Cer). 0.9% NaCl was used as vehicle control. **B**. Quantification of the replication markers Ki67 and pH3 expressed in pancreatic acinar (Ac) and interstitial (Int) cells after three days of 5-HTP supplementation and pancreatitis induction. **C**. Quantification of PU.1 expressing cells infiltrating the pancreas after three days of 5-HTP supplementation and pancreatitis induction. **D**. qPCR of acinar, ductal and progenitor markers after three days of 5-HTP supplementation and pancreatitis induction. **E**. qPCR of inflammation markers after three days of 5-HTP supplementation and pancreatitis induction. **F**. qPCR of inflammation and fibrosis markers after 14 days of 5-HTP supplementation and pancreatitis induction. **G**. Quantification of the replication marker Ki67 expressed in pancreatic acinar cells after 14 days of 5-HTP supplementation and pancreatitis induction. **H**. Quantification of ADM lesions after 14 days of 5-HTP supplementation and pancreatitis induction. **I**. Quantification of PU.1 expressing cells infiltrating the pancreas 14 days of 5-HTP supplementation and pancreatitis induction. Results are average ± SEM (n=5), *p<0.05.

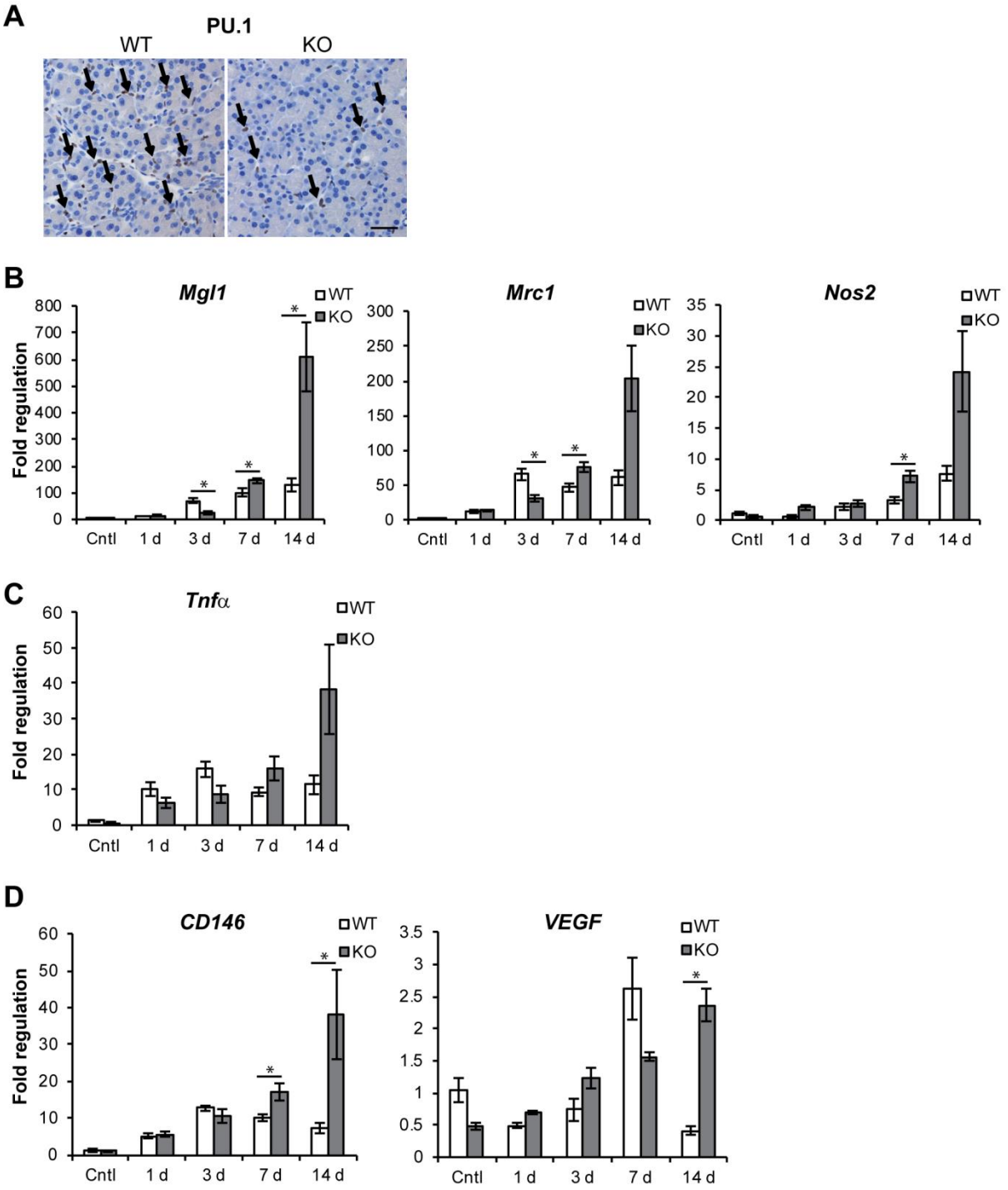

Figure S6. **A**. PU.1 immunostaining in WT and *Tph1*$^{-/-}$ pancreata three days after pancreatitis induction. **B**. qPCR of macrophage markers during the course of pancreatitis induction. **C**. qPCR of *Tnfα* during the course of pancreatitis induction. **D**. qPCR of vascular related factors during the course of pancreatitis induction. Results are average ± SEM (n=5), *p<0.05. Scale bars: 50 μm.

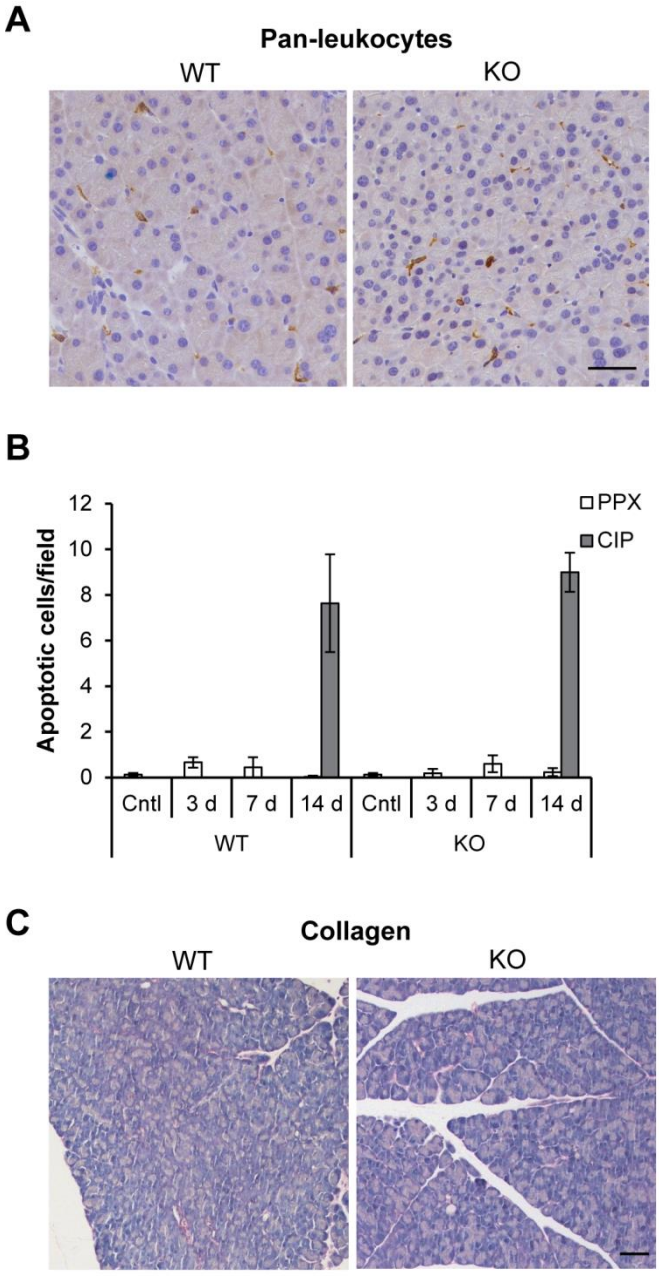

Figure S7. **A**. Pan-leukocyte immunostaining with anti-coronin-1 antibody two weeks after PPX. **B** TUNEL staining showed comparable number of apoptotic cells in intact acini in both strains at the indicated times following PPX. **C**. Sirius red staining showed comparable collagen deposition in the two strains two weeks after PPX. Scale bars: 50 μm.

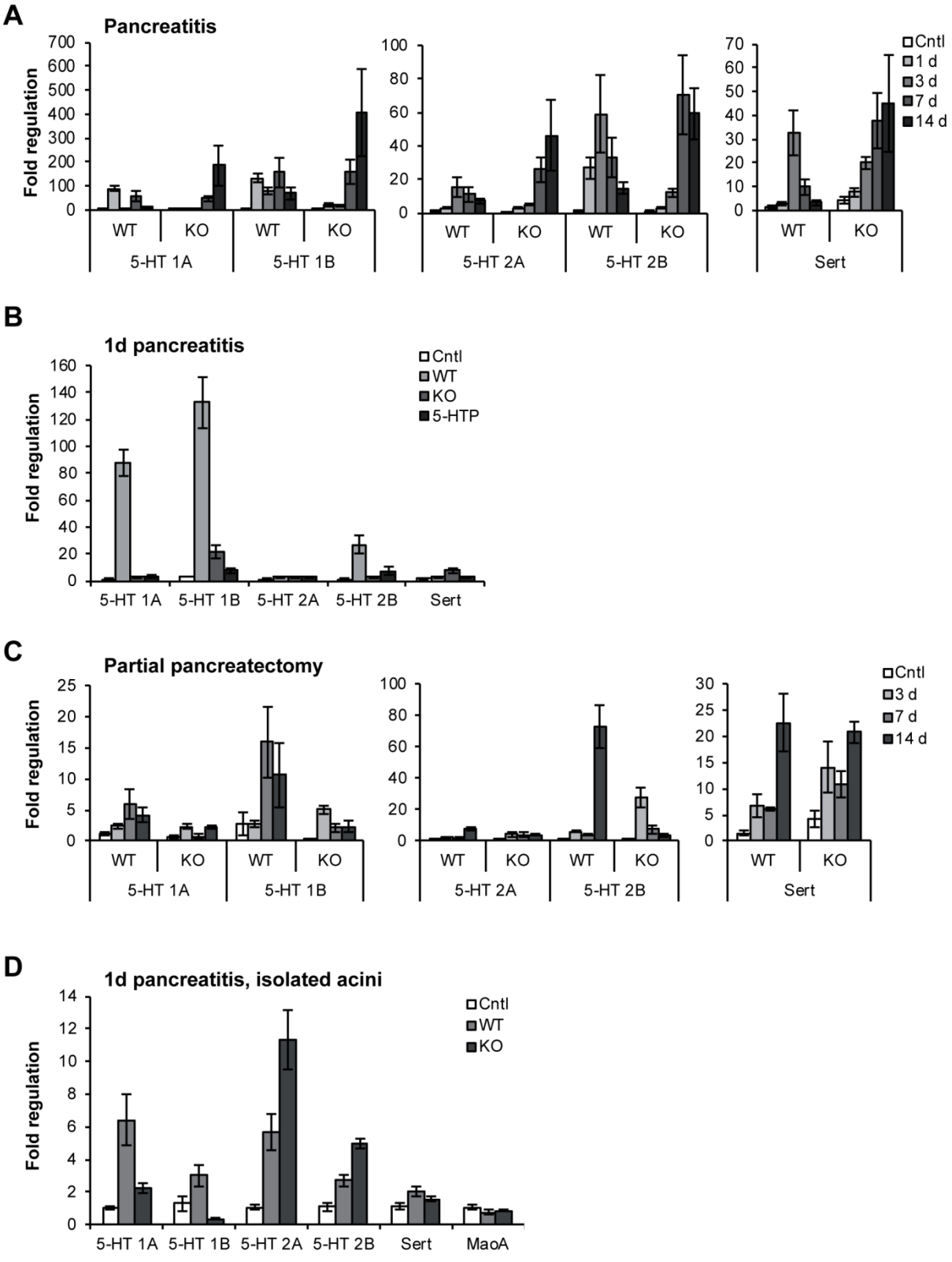

Figure S8. **A**. qPCR of 5-HT receptors and transporter in WT and *Tph1*$^{-/-}$ pancreata during the course of pancreatitis. **B**. qPCR of 5-HT receptors and transporter one day after pancreatitis induction in WT, *Tph1*$^{-/-}$ and WT mice supplemented with 5-HTP. **C**. qPCR of 5-HT receptors and transporter in WT and *Tph1*$^{-/-}$ pancreata following pancreatectomy. **D**. qPCR of 5-HT receptors and transporter and 5-HT deaminase enzyme monoaminooxidase A (MaoA) in acini isolated from WT and *Tph1*$^{-/-}$ mice one day after pancreatitis induction

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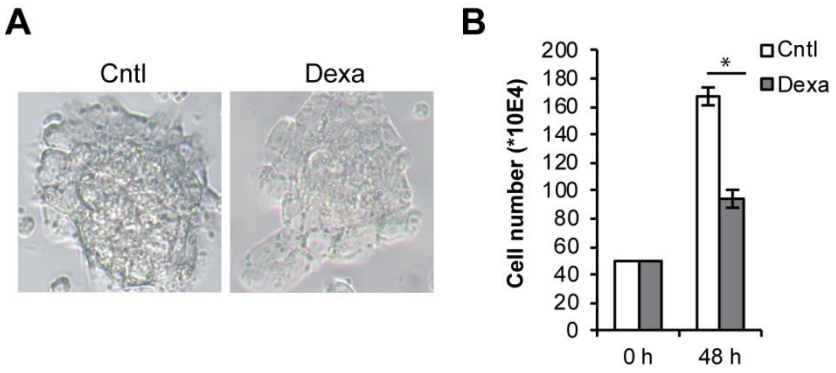

Figure S9. **A**. Bright field images of AR42J cells incubated for 2 days with dexamethasone. Note as the cells change morphology upon treatment. **B**. Quantification of AR42J cell number following 48 hours incubation with 50 nM dexamethasone (Dexa). Results are average ± SEM (n=3).

7. Discussion

5-HT has been recognized for decades as an important signaling molecule both in neuronal and non-neuronal processes, but its potential is possibly greater than ever expected. Novel emerging roles are constantly ascribed to 5-HT both in physiological and pathological conditions and, surprisingly, new intracellular mechanisms of action are now attributed to this multifunctional bioactive amine.

7.1 The role of 5-HT in cell secretion

The serotonergic network plays in fact a central role in nearly all cell types with an active secretory process. The term “network” has not been chosen accidentally: an increasing number of studies reports that the manipulation of one or more components related to the 5-HT system significantly alter the secretory activity both in a positive and a negative manner.

One of the best characterized examples in this sense are the mammary glands, which are influenced by 5-HT not only during development and homeostasis, but also substantially during lactation and the following glandular involution state. Of note, the initiating event for secretory activation requires cytoskeletal remodeling in which tight junctions assemble to ensure tissue integrity. During pregnancy and lactation, a physiological period in which mammary cells suffer a massive stress due to elevated protein synthesis and secretion, the hormone prolactin induces a significant increase of Tph1 mRNA levels in the glands. This increased gene expression reflects the local 5-HT production, which negatively controls milk production and secretion. Experiments using the $Tph1^{-/-}$ mouse model showed that 5-HT-deficient mice are hyper-responsive to prolactin and consequently secrete earlier and more abundantly milk compared to the wild type dames. The opposite outcome is obtained after interfering with 5-HT clearance, both by inhibiting SERT-mediated uptake and MAO activity. In this case milk production is prematurely interrupted and the glandular remodeling is significantly impaired leading to an involution-like state [66]. It is now proposed that 5-HT regulates mammary gland secretion according to a paracrine signaling pathway: the locally produced 5-HT accumulates in the interstitial fluid surrounding the glands and activates the 5-HT_7 receptors localized on the epithelial mammary cells. 5-HT_7 intracellular signaling negatively regulates milk protein synthesis and induces the disruption of tight junctions allowing glandular involution [67].

Epithelial mammary cells, similar to pancreatic β-cells described before in this thesis (chapter 3.6), constitute the perfect example of highly secretory cells responsive to physiological hormonal changes that require the entire 5-HT network to control their morphology and activities. Importantly, in both cases a cell-autonomous regulation it has been identified totally independent both from neuronal and platelet-derived 5-HT signaling.

Exocrine cells, situated in different body locations, share many similarities in terms of development, polarity and functions [68] and it wouldn't be surprising if they would share certain regulatory mechanisms.

Pancreatic exocrine acinar cells have been intensively investigated during the last decades and 5-HT was found to modulate zymogen secretion upon a receptor-mediated neuronal signaling. However, the possibility that non-neuronal 5-HT could regulate the secretion of these cells was not addressed so far.

In order to investigate this hypothesis, our group took advantage of a general Tph1$^{-/-}$ mouse model depleted of peripheral 5-HT but unaffected in the central nervous system. These animals do not show any pancreatic abnormalities. However, when we closer analyzed isolated acinar cells, we noticed that unstimulated Tph1$^{-/-}$ acini contained more zymogen positive granules compared to the WT acini. Further ultrastructural analysis with electron microscopy on pancreatic tissue confirmed that zymogen granules did not differ in terms of size within the strains but rather they were numerically more abundant and tightly packed in Tph1$^{-/-}$ mice. In addition, utilizing freshly isolated acini, currently considered a valid *ex vivo* experimental approach to investigate secretory mechanisms [69], we found that while basal secretion was comparable in the two strains, Tph1$^{-/-}$ acini secreted less zymogens when stimulated both at physiological and supramaximal concentration of the secretagogue cerulein. On the other hand, we also observed a block of both basal and cerulein-induced secretion when the rat acinar cell line AR42J was pre-incubated with a highly selective SERT inhibitor (fluoxetine), and with different antagonists of 5-HT receptor subtypes. To our surprise, SERT inhibition completely abolished the baso-lateral blebs-formation induced by actin cytoskeletal remodeling following supramaximal doses of cerulein [59]. Hence, our results indicate that 5-HT is required for the correct basal and stimulated secretion, possibly regulating cytoskeletal dynamics.

Given the central role of acinar cell secretion in the etiopathogenesis of acute pancreatitis, and supported by the evidence that 5-HT is involved in zymogen secretion, we explored the possibility that 5-HT modulates granule dynamics also *in vivo* in a pathological context, treating WT and Tph1$^{-/-}$ mice with high doses of cerulein. Cerulein administration represents the best model for clinical interstitial acute pancreatitis and it induces aberrant acinar secretion with consequent zymogen release into the interstitial space and eventually in the blood circulation. Eight hours after cerulein stimulation, we found a significant reduction of amylase in the Tph1$^{-/-}$ animal serum, symptom of a reduced interstitial zymogen secretion. Also in this case electron microscopy helped us to clarify how granules distribute upon stimulation. In both strains cerulein triggered a general enlargement of granule size, indicative of granule swelling preceding content expulsion. However, Tph1$^{-/-}$ granules were more packed, more abundant and bigger compared to the WT, presumably as a

consequence of impaired secretion and not due to zymogen synthesis. When we analyzed the cytoskeletal reorganization, known to be the driving mechanism for cell secretion [59-61], we observed a lack of baso-lateral actin re-organization and a different GTPase Rac1 localization.

Finally, in Tph1$^{-/-}$ animals the impairment of the secretory process led to a different onset and progression of the inflammatory phase. While in WT animals, 24 hours after the initiation of tissue insult, elevated levels of circulating zymogens were still detectable in blood and a clear immune response characterized the site of injury, in Tph1$^{-/-}$ animals blood parameters were found in baseline levels and the pancreatic tissue was nearly devoid of inflammation. Thus, our data strongly support the hypothesis that 5-HT-dependent ectopic secretion is needed for the initiation of pancreatitis.

Further interesting results were obtained when both animal strains were supplemented with 5-hydroxytryptophan (5-HTP), the precursor molecule converted into 5-HT. While in Tph1$^{-/-}$ animals 5-HTP supplementation, as expected, restored all the early hallmarks of pancreatitis, including elevated blood amylase and local inflammation, in WT animals the 5-HTP significantly attenuated the inflammatory phase.

5-HTP was recently found to stimulate, in a dose-dependent manner, acinar cell apical secretion and thus it has been associated with a protective effect during the initiation of acute pancreatitis [70]. Also in our experimental conditions, WT mice pre-treated with 5-HTP, displayed lower levels of inflammatory infiltrates, chemokine expression and serum amylase upon cerulein stimulation, presumably as a consequence of increased zymogen apical secretion into the duct thus counteracting the aberrant baso-lateral one.

7.2 The role of 5-HT in inflammation

There is now abundant evidence to suggest that 5-HT modulates the immune response and, in particular, inflammatory diseases. When injury occurs, mast cells and platelets release their 5-HT content, which in turn recruits and activates immune cells at the site of damage. Neutrophils, eosinophils, monocytes, macrophages, dendritic cells (DCs), mast cells and natural killer cells (NK) express a variety of 5-HT receptors. For some of these cell types, such as monocyte, macrophages and mast cells, the expression of SERT has been reported [71]. While the role of 5-HT uptake remains ambiguous and yet to be addressed, 5-HT receptor signaling is well known to work as an important modulator of chemotaxis and cytokine production. Inhibitors of 5-HT receptor class 1 and 2A, as well as agonists for receptor class 2B, 3 and 7 significantly attenuate several types of experimentally-induced inflammation, not only in the gut, but also in the liver and lungs. Additionally lack or irreversible inhibition of Tph1 enzyme delays and reduces the severity of different types of experimental colitis (reviewed in [72]).

Therefore, although our data strongly support the evidence that 5-HT modulates acinar cell secretion, we cannot exclude the possibility that other mechanisms, such as modulation of inflammation, may occur in Tph1$^{-/-}$ mice. In fact, eight hours from the first cerulein injection, a moderate serum amylase was detectable in Tph1$^{-/-}$ mice indicating that, even if reduced, ectopic secretion was taking place also in these mice. In this context, one would expect a reduced inflammatory cell influx into the tissue, but this was not the case as Tph1$^{-/-}$ mice that did not present any noticeable sign of inflammation in the pancreas after 24 hours of cerulein administration. Interestingly, also 5-HTP supplementation was associated with different inflammatory responses. Early 5-HTP supplementation in WT animals, prevented the onset of pancreatitis, while 2 weeks 5-HTP exposure, in combination with cerulein administration, culminated into a more severe local inflammation. The possible explanation for this dual effect may lie in 5-HTP metabolism. 5-HTP is quickly converted to 5-HT, which can be further metabolized in melatonin. Both 5-HTP and melatonin possess potent anti-inflammatory properties [73-75] and thus it cannot be excluded that during the early phase of pancreatitis, excess of 5-HTP, or a possible increase of melatonin, may be responsible for the observed anti-inflammatory effect. On the other hand, prolonged exposure to 5-HTP was associated to a more severe inflammation, possibly due to a preferential conversion of 5-HTP into 5-HT, well known for its pro-inflammatory properties. Future analysis on 5-HTP and its metabolites are necessary to better understand their role during the course of pancreatitis.

Hence, briefly summarizing what has been discussed so far, we could conclude that the 5-HT network is indispensable for patho-physiological zymogen secretion of acinar cells and that it modulates the immune response during the progression of pancreatitis. It remains now to be further discussed whether 5-HT influences also the tissue regeneration.

7.3 The role of 5-HT in tissue regeneration

5-HT has been considered for a long time a potent mitogenic factor and evidence of this role was observed in liver regeneration following hepatectomy, megakaryocyte colony formation, pulmonary artery smooth muscle cell proliferation and, as aforementioned, β cell expansion during pregnancy [44, 76-78].

In case of the adult pancreas, the mitogenic role of 5-HT in exocrine acinar cell proliferation and self-renewal was never addressed before. For the first time we reported that 5-HT does not mediate acinar cell proliferation in the absence of a strong inflammatory scenario. In fact, cell proliferation was not affected in Tph1$^{-/-}$ mice following 60% pancreatectomy, where mild inflammation is normally observed at the resection margin but not in the intact tissue. Corroborating data, in this sense, was obtained by supplementing both WT and Tph1$^{-/-}$ with the thyroid hormone T3, known to have a potent mitogenic effect on the pancreas [79]. Also in this case, lack of 5-HT did not interfere with cell proliferation, which was rather comparable

in the two strains, indicating that acinar cells, if not injured, are able to proliferate also in the absence of peripheral 5-HT.

Conversely, we discovered that peripheral 5-HT is instead critical in the context of cerulein-induced acinar injury and development of pancreatitis. Recent studies suggest that acinar cells may act as facultative progenitors capable of reverting to embryonic-like cells under injury conditions associated with inflammation. During this reprogramming phase, a complete down-regulation of acinar-specific genes is observed, in favor of the up-regulation of progenitor and ductal markers, such as Sox9 and CK19. Afterwards, a morphological remodeling of the acinar architecture to the duct-like structures, named ADMs, appears in the tissue. In WT animals, this process is transient and resolves in a relative short time, together with the overall morphology and functionality of the injured organ. In contrast, in Tph1$^{-/-}$ animals, all the mentioned consecutive steps resulted rather delayed and aberrant. Similarly to WT, the mutant mice displayed a prompt down-regulation of acinar-specific genes but this was not followed by acinar cell zymogen emptying as observed in the WT cells. Thus, the subsequent up-regulation of ductal markers as well as the appearance of ADMs was postponed in the mutant mice concomitant with the delayed onset of inflammation. Furthermore, it is important to remark that only the secretory process but not zymogen synthesis or their activation was compromised in Tph1$^{-/-}$ mice. Therefore, when the harmful cargo was finally secreted, it probably culminated into a more severe and sustained inflammation compared to WT mice. This hypothesis is supported by the exceptional up-regulation of macrophage markers accompanied with more abundant acinar cell proliferation and ADM formation in Tph1$^{-/-}$ mice. Therefore, our experimental approaches support the correlation between acinar trans-differentiation and the type of tissue injury, which confirms the pivotal role of the local activation of innate cells.

To conclude, the physical removal of intracellular digestive zymogens, via a 5-HT dependent secretion, constitutes one of the early but fundamental processes initiating the cascade of events characterizing the onset of pancreatitis.

8. Mouse model limitations

The mouse model utilized in our experimental studies revealed a significant role of 5-HT in the etiopathogenesis of pancreatitis. However, this has several limitations.

Firstly, a general tph1 knocked out system is unsuitable to define which cell type constitutes the main source of 5-HT production to guarantee pancreatic acinar cell secretion. Secondly, lack of tph1 but not tph2 implies the possibility that a certain undefined amount of 5-HT is locally released by enteric neuronal cells, thus our system was not completely devoid of 5-HT. Neuronal 5-HT stimulus, for instance, would explain why a reduced acinar cell secretion was still observed *in vivo* but not *ex vivo* in Tph1$^{-/-}$ mice. Finally, as extensively discussed in

this thesis, the three main processes observed during the course of pancreatitis, namely cell secretion, tissue inflammation and acinar cell regeneration, are directly and indirectly linked with the 5-HT network. Therefore, modulation of cell specific 5-HT synthesis, receptor signaling, intracellular uptake, biochemical precursor availability and metabolism is needed to elucidate the role of 5-HT in each cell type taking part to the process.

9. Future investigations

Adult acinar cells acquire plastic characteristics losing their cellular identity in response to inflammation-mediated injury. This instability creates a window where a cell can either be redirected into a differentiated state, or undergo deregulated neoplasia in the presence of oncogene expression. ADMs represent the critical step influencing these two fates. During the recovery phase of acute pancreatitis, ADMs represent a pool of cells with exceptional plasticity required for tissue regeneration.

For a long time acinar cells were suspected to actively contribute to pancreatic tumorigenesis, but a definitive confirmation derives from recent lineage-tracing studies showing that persistent ADM lesions may lead to pre-malignant pancreatic lesions [80].

Noteworthy, ADM transition depends on cytoskeletal remodeling of acinar cells, but the molecular mechanisms regulating this process are not completely elucidated. In the last few years the small GTPase Rac1 was shown to be responsible for the cytoskeletal rearrangements necessary for ADM formation. Specifically, activation of Rac1 drives the re-localization of actin filaments from the apical to the baso-lateral side of trans-differentiating acinar cells, a sign for loss of cell polarity [81]. Importantly, this actin remodeling is similar to the one found to induce the aberrant baso-lateral zymogen secretion typically observed upon supramaximal stimulation with cerulein [59]. Finally, Rac1 was also identified as a proto-oncogene responsible for pancreatic ductal adenocarcinoma (PDAC) progression due to its involvement in promoting cell motility and metastasis [82, 83]. Our group demonstrated that 5-HT regulates zymogen secretion of pancreatic acinar cells following cerulein-induced pancreatitis, influences actin relocalization during the acute phase of the disease, and it is fundamental for ADM formation during the recovery phase.

Based on multiple evidence indicating that 5-HT influences several processes otherwise attributable to Rac1, we intend now to investigate whether a functional correlation within these two molecules occurs during ADM formation. Rac1 belongs to the Rho family of small G proteins, a subgroup of the Ras superfamily and regulates a variety of cellular processes, such as cytoskeletal organization, vesicular transport, cell cycle progression, cell adhesion and migration, neuronal differentiation, and several enzymatic activities [84, 85]. Rac1 functions depend on a cycle of molecular switch between two conformational states: a GTP-bound, active state and a GDP-bound, inactive state. The GTP/GDP cycling is controlled by

many regulatory molecules, such as GTPase activating proteins (GAPs), guanine nucleotide exchange factors (GEFs) and GDP dissociation inhibitors (GDIs) [86]. A number of post-translation modifications are also found both in the regulator-interaction sites and in the GTP-hydrolyzing domain greatly affecting the GTPase activation cycle. One of the possible modifications consists in the transamidation of primary amines on the glutamine residues of GTPases. 5-HT constitutes one of the intracellular sources of amines. Transglutaminases mediate the cross-linking reaction between the amine and protein residues and the resulting modification is named *serotonylation*. Moreover, this mechanism depends on the SERT-mediated 5-HT uptake in conjunction with Ca^{2+} mobilization. Notably, Rac1 activity was demonstrated to be transiently increased due to TGase-mediated serotonylation of Rac1 via stimulation of $5\text{-}HT_{2A}$ receptors (reviewed in [18, 87]).

10. Preliminary results

10.1 Acinar cells uptake 5-HT via SERT

As described in the previous paragraph, SERT-mediated 5-HT uptake is necessary to modulate GTPases activity, implying the important role of intracellular 5-HT in this mechanism. Before investigating whether 5-HT modulates the activity of GTPases, it was fundamental to prove that a SERT-mediated 5-HT uptake occurs also in pancreatic acinar cells. For this reason, an [3H]-5-hydroxytryptamine-uptake assay was performed both in freshly isolated murine acinar cells and in rat AR42J cell line. Two independent experiments, in both cellular systems, clearly indicated that acinar cells are able to uptake 5-HT via an active transport mechanism and that this transport is significantly reduced by fluoxetine, a highly specific SERT inhibitor (Fig.6).

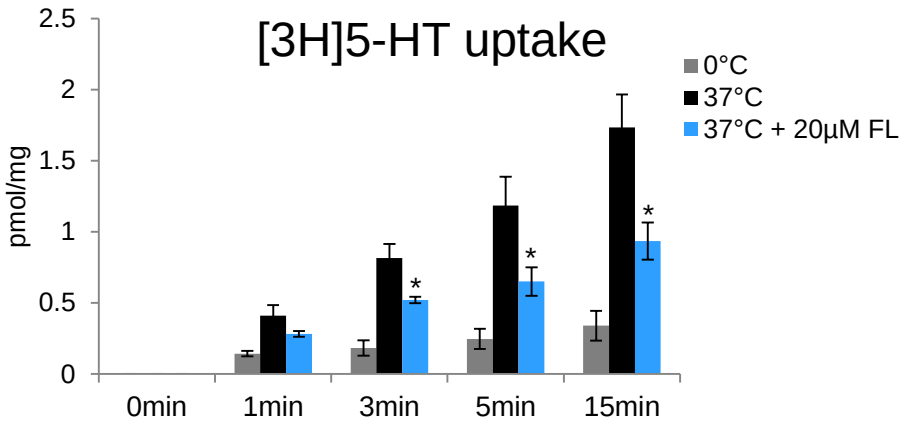

Figure 6. Fluoxetine prevents 5-HT uptake in pancreatic acinar cells. The time-course was performed at 37°C in presence or absence of 20µM FL and at 0°C to determine non-specific uptake. Note the significant 5-HT uptake inhibition at 37°C (active transport) in presence of fluoxetine (FL).

10.2 SERT inhibition prevents Rac1 activation *in vitro*

Given that serotonylation increases the activity of Rac1, we took advantage of an elegant FRET-based assay to test whether limiting the intracellular 5-HT availability decreased Rac1 activation.

Five independent experiments performed on the AR42J cell line demonstrated that inhibition of SERT with fluoxetine was sufficient to prevent Rac1 activation (Fig.7), suggesting that reducing intracellular levels of 5-HT has the potential to inhibit the activity of proteins that are targets of serotonylation.

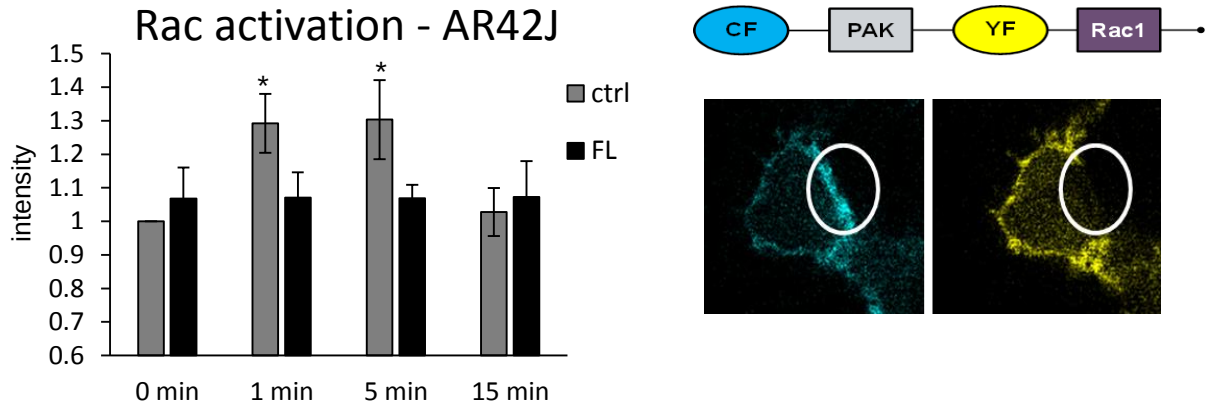

Figure 7. Inhibition of 5-HT uptake prevents Rac1 activation. Left panel, time course quantification of Rac1 activation in presence of the SERT inhibitor fluoxetine (black columns), as determined by FRET assay. FRET was performed using the donor recovery after acceptor photo-bleaching method using the FRET-AB wizard of the Leica software. Acceptor (yellow fluorescent protein) fluorescence was bleached at 100% laser intensity. Right panel, schematic representation of the used construct and representative confocal image.

10.3 SERT inhibition prevents ADM formation *in vitro*

Given that ADM formation is tightly dependent on cytoskeletal rearrangements driven by the activation of Rac1 and that Rac1 activity is reduced *in vitro* upon SERT inhibition with fluoxetine, we tested whether fluoxetine treatment prevented Rac1-driven ADM formation in a 3D culture system. Pancreatic explants from mice harboring the $Kras^{G12D}$ mutation, sufficient to induce ADM formation both *in vivo* and in 3D culture [88], were used for this purpose. Rac1 inhibitor (iRac1) was used as a positive control, while TG inhibitor (cysteamine, Cys) was used to test whether TG-dependent serotonylation occurred during ADM formation. Cys and iRac1 significantly reduced ADM formation, but fluoxetine treatment completely prevented their appearance (Fig.8).

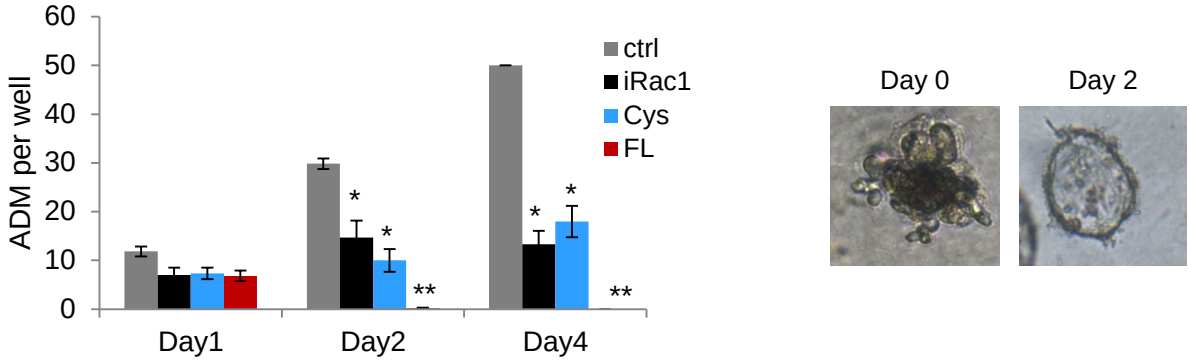

Figure 8. Inhibition of Rac1, TG and 5-HT uptake inhibits ADM formation in 3D cultures of pancreatic explants. Explants were embedded in collagen, treated with the SERT inhibitor fluoxetine (FL, red columns) and the number of ADM quantified after one and two days of culture. Right panel, representative images of acinar clusters (Day0) and ADM (Day 2).

Collectively, these data suggest that 5-HT promotes cytoskeletal remodeling underlying the formation of ADM lesions. Present and future work aim to investigate whether inhibition of 5-HT uptake affects ADM formation in a pancreatic cancer model and thus might represent a valid therapeutic approach to prevent spreading of pre-malignant lesions.

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2000-2005: Liceo scientifico “E.Majorana”, Putignano (Ba), Italy. Maturity certificate

Additional Training

2015: Advance course in transcriptomic and genomic analysis, FGCZ, Zurich, Switzerland
2015: Advance scientific grant writing, Giessbach, Brienz, Switzerland
2014: UEG Basic Science Course on “Hot topics in experimental GI cancer”, Munich, Germany
2012: Course in Laboratory Animal Science, FELASA, Zurich, Switzerland
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Publications

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